Class I_A Phosphatidylinositol 3-Kinase p110 Alpha: a Critical Mediator for Cell Proliferation, Survival and Tumour Formation in Small Cell Lung Cancer and Embryonal Tumours

Dissertation
zur
Erlangung der naturwissenschaftlichen Doktorwürde
(Dr. sc. nat.)
vorgelegt der
Mathematisch-naturwissenschaftlichen Fakultät
der
Universität Zürich
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Zürich, 2012

The experimental work presented in this thesis was performed at the Division of Oncology at the Children's University Hospital Zurich and at the Department of Clinical Research of the University of Bern. The supervision of this thesis was conducted by PD Dr. Alexandre Arcaro (Department Klinische Forschung, Abteilung für Pädiatrische Hämatologie/Onkologie, Universität Bern), Prof. Dr. Ruedi Aebersold (Institute of Molecular Systems Biology, ETH Zurich), and Prof. Dr. Uwe Zangemeister-Wittke (Institute of Pharmacology, University of Bern).

Zurich, 2012
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1 SUMMARY

The PI3K signalling pathway is known to be fundamental for cell proliferation, growth, and survival, and to contribute to cancer development. Its deregulation and contribution to carcinogenesis has been well documented and reviewed in the past. PI3Ks are downstream effectors of receptor tyrosine kinases located at the cellular membrane, which play a central role in the regulation of cellular activities including cell proliferation and survival, growth, cell cycle control, metabolism, cell adhesion, and motility. Targeting different components of the RTK/PI3K pathway with pharmacological inhibitors has already been shown to be a promising approach in cancer treatment. Inhibitors of the RTK/PI3K pathway have reached the clinical stage in some cases and the development of new small molecule inhibitors is still an ongoing process.

Lung cancer is the leading cause of cancer-related mortality in the Western world and every year approximately 1.4 million people are diagnosed with lung cancer. Small cell lung cancer is the most aggressive of all lung cancer types, accounts for approximately 15% of all lung cancer cases and is almost entirely related to tobacco smoking. It is characterized by rapid growth and early metastasis. Although SCLC initially responds to therapy most of the patients relapse. Due to metastasis and resistance to chemotherapy the clinical outcome is very poor and SCLC patients show an overall 5-year survival of less than 5%. Consequently, novel therapeutic strategies are urgently required for SCLC. The molecular mechanisms responsible for lung cancer formation have been extensively studied. A striking event in the development of cancer is the escape of atypical cells from the normal growth control and their transformation into a malignant and invasive phenotype. Numerous genetic and molecular alterations have been reported to be associated with the development of SCLC, including autocrine signalling loops, oncogene activation and loss of tumour-suppressor genes. These genetic and molecular aberrations result in a lack of response to negative growth regulatory signals and the continuous presence of positive signals that regulate growth, motility, and invasion.

The PI3K/Akt/mTOR pathway has been demonstrated to play a key role in SCLC cell proliferation, survival, chemoresistance and migration. Mutations in *PIK3CA* and gene amplification were reported in primary SCLC, as well as increased expression of *PIK3CA* at the mRNA and protein level. Broad specificity PI3K/mTOR inhibitors have shown anti-tumour activity in SCLC models *in vitro* and *in vivo*.

Thus, we sought to investigate the particular role of the class I_A PI3K isoform p110α and the potential of targeting class I_A PI3Ks with isoform-specific inhibitors and RNAi.

SCLC patient samples displayed over-expression of p110α and p110β, and increased over-expression of p110α correlated with advanced-staged SCLC, which was not the case for p110β.

Targeting p110α by RNAi or isoform-specific inhibitors had more pronounced effects on SCLC cell responses, than in the case of p110β or p110δ, indicating a selective role for p110α in SCLC. We found impaired cell viability and activation status of classical PI3K downstream targets, such as Akt, S6 and 4EBP1, as well as decreased transcript levels of the pro-angiogenic marker VEGFA.

Furthermore, we hypothesized that p110α may control the expression of a selective subset of genes implicated in SCLC cell proliferation and/or survival. Indeed, targeting p110α affected the expression of the Bcl2 family of proteins at the transcript and protein level. Importantly, the Bcl-2 family of proteins has been previously shown to play a crucial role in the survival of SCLC cell lines *in vitro* and *in vivo.* In the present study, pro-apoptotic proteins, such as Bad and Bax, were found to be up-regulated, whereas the anti-apoptotic Bcl2 and $BclX_L$ were down-regulated. Additionally, the p110α inhibitors induced increases in both SCLC apoptosis and autophagy, which is consistent with Bcl2 being a target of p110α. Bcl-2 is a key regulator of both apoptosis and autophagy, and its expression is controlled by the transcription factor NFκB. In fact, we found deregulation in the NFκB transcriptional network and reduced protein expression upon p110α inhibition. Its specific role in SCLC in view of its association with p110α and the Bcl2 family proteins appears to be an interesting topic for future research.

Finally, we confirmed our results *in vivo*. SCLC tumour formation was impaired by p110α inhibition and accompanied by affected vascularization.

In summary, we demonstrated that the class I_A PI3K isoform p110α is a critical mediator controlling SCLC tumour growth, proliferation, and survival processes.

In a second project we evaluated the potential of targeting the IGF-IR/PI3K signalling axis in the embryonal tumours neuroblastoma, the most common extracranial tumour in children and medulloblastoma, the most frequent childhood brain tumour.

Activation and deregulation of the IGF-IR/PI3K signalling system has been reported to be present in NB and MB, including autocrine signalling loops, over-expression and genetic alteration found in genes encoding PI3K signalling components.

By treating NB and MB cells with R1507 (Roche), a specific humanised monoclonal antibody against the IGF-IR, or targeting the class I_A PI3K p110α with the specific inhibitor PIK75, we wanted to gain knowledge about their impact on cell viability, survival, expression and phosphorylation status of IGF-IR/PI3K downstream signalling targets, as well as their effects on chemoresistance. We observed cell line-specific responses and a generally larger number of NB and MB cell lines responded to PIK75 than to R1507. This might be due to PI3K activation downstream of numerous RTKs expressed in NB and MB, suggesting that combinatorial approaches of RTK inhibition and classical chemotherapeutic treatments are superior to targeting one single RTK. In addition, PI3K isoforms appear to be targets of choice for further drug development.

In the present study, we demonstrated that targeting the class I PI3K isoform p110α in embryonal tumours clearly has advantages as an anti-cancer approach over the inhibition of the IGF-IR *in vitro*.

2 ZUSAMMENFASSUNG

Die Phosphatidylinositol-3-Kinase-(PI3K)-Signaltransduktionkaskade spielt eine elementare Rolle in der Zellproliferation, dem Zellwachstum und ihrem Überleben, ist jedoch auch bekannt dafür, karzinogene Prozesse zu fördern. Deregulierung innerhalb des PI3K-Signaltransduktionswegs und sein Beitrag zur Tumorgenese wurden in der Vergangenheit bereits oft dokumentiert und diskutiert. PI3K sind Rezeptortyrosinkinasen (RTK) nachgeschaltete, an der zellulären Plasmamembran befindliche Effektormoleküle, die eine zentrale Rolle in der Regulation zellulärer Prozesse übernehmen. Das Targeting unterschiedlicher Komponenten des RTK/PI3K-Signalwegs mit pharmakologischen Inhibitoren wurde bereits als vielversprechender Ansatz für die Krebsbehandlung dargestellt. Inhibitoren der RTK/PI3K-Signalkaskade haben in einigen Fällen die klinische Phase der Forschung erreicht, und die Entwicklung neuartiger „small-molecule"-Inhibitoren dauert an.

Lungenkrebs ist die führende Ursache der Krebs-zugrundeliegenden Sterblichkeit in der westlichen Welt. Etwa 1.4 Mio Menschen sind jährlich von der Diagnose Lungenkrebs betroffen. Das kleinzellige Lungenkarzinom (SCLC), der aggressivste aller Lungenkrebsarten, beläuft sich auf etwa 15% der Lungenkrebsfälle und ist beinahe vollständig auf Zigarettenkonsum zurückzuführen. SCLC ist durch extrem schnelles Wachstum und frühzeitige Metastasierung gekennzeichnet. Obwohl SCLC zunächst auf die angewandten Therapien anspricht, erleiden die meisten Patienten einen Rückfall. Der schlechte klinische Verlauf entsteht durch Metastasierung und Chemotherapie-Resistenzen; SCLC-Patienten zeigen eine 5-Jahres-Überlebenrate von unter 5%. Demzufolge werden neuartige Strategien für die Behandlung von SCLC dringend benötigt. Die molekularen Mechanismen, die der Entwicklung von Lungenkrebs zugrunde liegen, wurden bereits intensiv untersucht. Ein augenscheinlich beachtliches Ereignis der Tumorgenese ist, dass sich atypische Zellen der zellulären Wachstumskontrolle entziehen und infolgedessen einen bösartigen und invasiven Phänotyp ausprägen.

Zahlreiche mit der Entwicklung des kleinzelligen Lungenkarzinoms zusammenhängende genetische und molekulare Veränderungen wurden bereits beschrieben, wie beispielsweise die autokrine Signalübertragung, die Aktivierung von Onkogenen und der Verlust von Tumorsuppressorgenen. Diese Abweichungen führen zu einer verminderten Zellantwort auf negative Wachstumssignale und die kontinuierliche Präsenz positiver Signale, die das Wachstum, die Motilität und die Fähigkeit zum invasiven Wachstum regulieren.

Der PI3K/Akt/mTOR-Signalkaskade wurde bereits eine Schlüsselrolle in der Zellproliferation, dem Zellüberleben, der Chemotherapie-Resistenz und der Migration von SCLC-Krebszellen nachgewiesen. Primäre SCLC-Tumore weisen Mutationen im Gen *PIK3CA* und Genamplifikationen sowie eine erhöhte Expression der PIK3CA-Transkripte und -Proteine auf. PI3K/mTOR-Inhibitoren mit breitgefächertem Wirkungsspektrum zeigten Antitumoreffekte in SCLC Modellen *in vitro* und *in vivo*.

Uns darauf beziehend, fokussierten wir in diesem Projekt die Rolle der Klasse-I_A-PI3K-Isoform p110α im kleinzelligen Lungenkarzinom und das Potenzial des Targetings von Klasse-I_A-PI3K mit RNA-Interferenz und Isoform-spezifischen Inhibitoren.

SCLC-Gewebeproben wiesen eine Überexpression von p110α und p110β auf. Im Gegensatz zu p110β korrelierte eine ansteigende p110α-Überexpression mit fortgeschrittenem SCLC.
Das Targeting von p110α zeigte einen deutlich stärkeren Effekt auf SCLC Zellantworten gegenüber dem Targeting von p110β und p110δ. Wir konnten eingeschränkte Zellviabilität und beeinträchtigten Aktivierungszustand verschiedener PI3K-nachgeschalteter Moleküle (PI3K-„Downstream-Moleküle“) feststellen, wie beispielsweise Akt, S6 und 4EBP1 sowie verminderte Transkriptlevel des Angiogenese-Faktors VEGFA.
Wir stellten die Hypothese auf, dass p110α die Genexpression in SCLC kontrollieren könne. In der Tat führte das Targeting von p110α zu einer Beeinträchtigung der Expression der Bcl2-Protein-Familie auf dem Transkript- und Proteinlevel. Es konnte bereits vorher gezeigt werden, dass die Bcl2-Protein-Familie eine elementare Rolle im Überleben von SCLC-Zellen spielt. Wir zeigten, dass proapoptotische Proteine, wie Bad und Bax, heraufreguliert wurden, während die antiapoptotischen Proteine Bcl2 und $BclX_L$ herunterreguliert waren. Zusätzlich induzierten die p110α Inhibitoren Apoptose und Autophagie, was unserer Annahme von Bcl2 als einem p110α-„Downstream-Target“ entspricht, da es sich bei Bcl2 um einen Schlüsselregulator von Apoptose und Autophagie handelt. Die Bcl2-Expression wird durch den Transkriptionsfaktor NFκB kontrolliert. Tatsächlich konnten wir zeigen, dass die Hemmung von p110α das transkriptionelle Netzwerk um NFκB deregulierte und die Expression des NFκB-Proteins verminderte. Die Rolle von NFκB als „p110α-Downstream-Target“ im Zusammenhang mit der Regulation der Bcl2-Proteine im kleinzelligen Lungenkarzinom erscheint ein interessantes Thema für die zukünftige Forschung zu sein.
Zu guter Letzt konnten wir unsere Ergebnisse in einem *in vivo* Versuch bestätigen. Das SCLC Wachstum konnte durch das Targeting von p110α gesenkt werden und war von beeinträchtigter Vaskularisierung begleitet.
Zusammenfassend konnten wir in der hier vorliegenden Studie zeigen, dass es sich bei der Klasse-I_A-PI3K-Isoform p110α um einen entscheidenden Signalmediator handelt, der SCLC Tumorwachstums-, Proliferations- und Zellüberlebensprozesse kontrolliert.

In einer weiteren Studie untersuchten wir das Potenzial des Targetings der IGF-IR/PI3K-Signalachse in zwei embryonalen Tumoren: Neuroblastom (NB), der am häufigsten auftretende extracranielle Tumor bei Kindern, und Medulloblastom (MB), der häufigste Hirntumor bei Kindern.
Aktivierung und Deregulierung des IGF-IR/PI3K-Signal-Systems konnten bereits in NB und MB gezeigt werden, einschließlich autokriner Signaltransduktion, Überexpression und genetischer Veränderungen in Genen, die für PI3K-Signalmoleküle kodieren.
Durch die Behandlung von NB- und MB-Zellen mit R1507 (Roche), einem monoklonalen IGF-IR-Antikörper oder PIK75, einem spezifischen Klasse-I_A-PI3K-Isoform-p110α-Inhibitor, erhofften wir uns Einsicht in die Wirkweise der Behandlung auf Zellviabilität, Aktivierungszustand verschiedener PI3K-„Downstream-Moleküle“ und ihren Effekt auf Chemoresistenz.

Wir beobachteten Zelllinien-spezifische Reaktionen und einen generell stärkeren Effekt von PIK75 im Vergleich zu R1507. Dieser könnte darauf zurückzuführen sein, dass die PI3K-Aktivierung vielen verschiedenen RTK nachgeschaltet ist. Dies führt zu der Annahme, dass Behandlungsansätze aus einer Kombination von RTK-Inhibitoren und klassischen Chemotherapeutika Vorteile gegenüber des alleinigen Targetings einer einzelnen RTK besitzen. Zusätzlich scheinen PI3K-Isoformen „Targets der Wahl“ für die weitere Medikamentenentwicklung zu sein.

In der hier vorliegenden *in vitro*-Studie konnten wir zeigen, dass wir mit dem Targeting der Klasse-I_A-PI3K-Isoform p110α in embryonalen Tumoren einen deutlich höheren Effekt auf Wachstums- und Überlebensprozesse embryonaler Tumorzellen erzielen konnten als mit dem Targeting von IGF-IR.

3 INTRODUCTION

3.1 PHOSPHATIDYLINOSITOL-3 KINASES

PI3Ks are a family of evolutionary conserved lipid kinases which phosphorylate the 3-hydroxyl group of the inositol ring of three species of phosphatidylinositol (PtdIns) lipid substrates in the plasma membrane of the cell: PtdIns, PtdIns-4-phosphate, and PtdIns-4,5-bisphosphate, thereby producing second messengers such as PtdIns(3)P (PI(3)P), PtdIns(3,4)P_2 (PI(3,4)P_2), and PtdIns(3,4,5)P_3 (PI(3,4,5)P_3) (1). These second messenger molecules recruit multiple target proteins involved in complex downstream signalling cascades to the plasma membrane, which bind these PtdIns through specific lipid-binding domains, namely pleckstrin homology (PH) domain, phox homology (PX) domain, and FYVE (Fab-1, YGL023, Vps27, and EEA1) zinc finger domain, resulting in initiation of signalling pathways providing proliferation, survival, and motility signals (2-4). The PI3K signalling pathway, fundamental for cell proliferation, growth, and survival, is known to play a crucial role in embryonic development and immunity, but is known as well to contribute to the development of major human diseases, such as cancer, inflammation, and diabetes. Its deregulation and contribution to carcinogenesis has been well documented and reviewed in the past (5). The PI3Ks are major downstream effectors of receptor tyrosine kinases (RTKs), which will be described below, and G protein-coupled receptors (GPCRs).

Central components of cell signalling networks are receptor tyrosine kinases (RTKs) located at the cellular membrane, playing a central role in the regulation of fundamental cellular activities including cell proliferation and survival, growth, cell cycle control, metabolism, cell adhesion, and motility. Approximately 60 RTKs have been identified, all sharing a similar structure (6). They are sub-classified according to primary structure, ligand affinity, and induction of biological response. RTKs contain an N-terminal extracellular domain for ligand binding, a single α-helix transmembrane domain, and a C-terminal domain with a juxtamembrane regulatory regions and tyrosine kinase activity located in the cellular cytoplasm. The majority of RTKs are presented as monomers (7) and activation upon ligand binding induces conformational alterations followed by dimerization of receptor subunits and leads to auto-phosphorylation of specific tyrosine residues within the cytoplasmic domains (8). The auto-phosphorylation of RTKs leads to recruitment and activation of a variety of signal transducers, which recognise phosphorylated tyrosine residues and bind via SH2 (Src homology 2) and PTB (phosphotyrosine binding) domains (9), either directly to the receptor or by binding to other adaptor and docking proteins, such as Grb2 and Shc, or IRS1 and Gab-1. These proteins act as interaction partners or as intermediates recruiting multiple signalling molecules, finally activating several downstream signalling cascades through important mediators, such as PI3K/Akt/mTOR or Ras/Raf/MEK/Erk, triggering signals to promote cell proliferation, prevent apoptosis, and increase cell migration. Dysfunctional RTK signalling has been shown to be critical for

the development and progression of many types of cancer and the involvement of RTKs in tumourigenesis has led to the design of new drugs that specifically inhibit RTK activity. Targeting different components of the RTK/PI3K pathway with pharmacological inhibitors or neutralizing antibodies has already been shown to be a promising approach in cancer treatment (5, 10, 11). Inhibitors of the RTK/PI3K pathway have reached the clinical stage in some cases and the development of new small molecule inhibitors is still on-going.

CLASSIFICATION OF PI3K FAMILY MEMBERS

The family of PI3Ks has been divided into three classes according to their structural characteristics and lipid substrate preferences and comprises 8 catalytic isoforms in human (12).

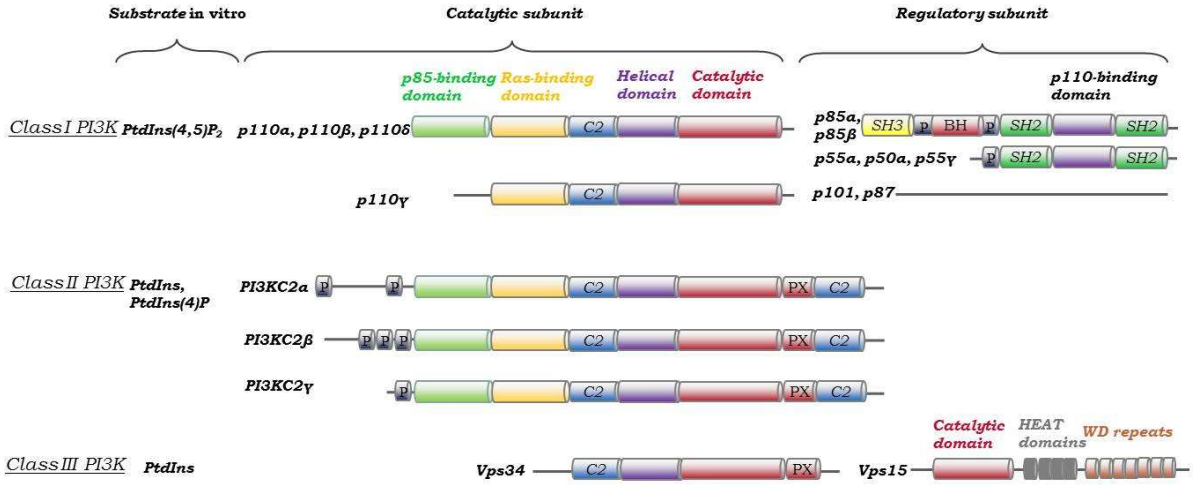

Figure 1. The Classification of the PI3K Family Members.

Class I PI3Ks. Class I PI3Ks are subdivided into class I_A (p110α, p110β, p110δ) and class I_B (p110γ) based on the type of receptor they are activated by, the regulatory subunits they are connecting, and their activation mechanism. They mainly generate the second messenger PtdIns(3,4,5)P_3 *in vivo* (1, 12). Class I_A PI3Ks are activated by RTKs (12) and certain oncogenes such as the small G protein Ras, and consist of heterodimers comprising a catalytic subunit and a regulatory subunit. In mammals, the catalytic p110 subunits are encoded by three genes: *PIK3CA*, *PIK3CB*, and *PIK3CD*. The catalytic p110 subunit possesses an N-terminal p85-binding domain, a Ras-binding domain that mediates Ras-dependent activation, a C2 domain, a phosphatidylinositol kinase homology (PIK) domain and a catalytic domain. The catalytic subunit undergoes constitutive association with one of the regulatory subunits p85α, p55α, p50α, p85β, or p55γ, which are generated by alternative splicing of the three genes *PIK3R1*, *PIK3R2* and *PIK3R3* (13-16). In response to growth factor-stimulated receptor activation the PI3K is recruited to the plasma membrane via direct interaction of its regulatory subunit with tyrosine phosphate residues of the activated receptor or associated adaptor proteins (via Src

homology-2 (SH2) domains) (1). Finally, the close proximity to its lipid substrates and the release of the basal inhibition of p110 through p85 is necessary to activate its catalytic activity. The class I_B PI3K is a heterodimer consisting of the unique catalytic subunit p110γ and its regulatory subunit p101 and is only found to be present in mammals. The Gβγ subunit of trimeric G proteins downstream of activated GPCRs binds to the regulatory subunit and in turn activates p110γ (17). Another regulatory subunit, named p84 (also $p87^{PIKAP}$) PI3K adaptor protein, have been identified (18). The PI3K class I_B signalling plays an important role in activation, growth and proliferation of immune cells (19). In mammals, class I PI3Ks are present in all cell types throughout the body (20), with p110δ and p110γ being highly enriched in leucocytes (19, 21). The role of the class I PI3Ks in human diseases is the best characterized among the three classes of PI3Ks.

Class II PI3Ks. Class II PI3Ks are monomers, consisting of a single catalytic subunit (PI3KC2α, PI3KC2β, PI3KC2γ) and lack a regulatory subunit. Preferentially, class II PI3Ks phosphorylate PtdIns and PtdIns(4)P and are activated by RTKs, cytokine receptors and integrins. Unlike the well-characterized class I PI3Ks, which have been extensively studied over the last 25 years, much less is known about the cellular functions of class II PI3Ks. Mammalian cell studies suggested they might have important roles in biological processes, such as cell migration, glucose metabolism, exocytosis, smooth muscle contraction, and apoptosis (22).

Class III PI3Ks. Class III PI3Ks were first described in *Saccharomyces cerevisae* and are heterodimers consisting of the regulatory subunit p150 (Vps15) and the catalytic subunit Vps34 (homologue of the yeast vacuolar protein sorting-associated protein 34). The only substrate they phosphorylate is PtdIns, generating PtdIns(3)P, which is an important regulator of membrane trafficking (23, 24). The class III PI3K has been shown to be a nutrient-dependent lipid kinase mediating signalling through mTOR, indicating a possible contribution to cell growth control processes (25, 26). Furthermore, it has been implicated to be involved in phagosome formation, transport at the nuclear membrane, and to be an important regulator for autophagy, the cellular response to nutrient starvation (27).

THE CLASS I_A PI3K SIGNALLING PATHWAY

Activation of PI3K is initiated by binding of various growth factors to their specific receptors. Class I_A PI3K are recruited to RTKs at the plasma membrane as heterodimers, consisting of a regulatory (p85) and a catalytic subunit (p110). The p85 regulatory subunit binds to phosphotyrosine residues in the cytoplasmic domain of RTKs through its Src-homology 2 (SH2) domains, which leads to PI3K activation (12, 28). As a consequence, the second messenger phosphatidylinositol-3,4,5-trisphosphate (PIP_3) is generated through phosphorylation of phosphatidylinositol-4,5-bisphosphate (PIP_2). A direct antagonist of PI3K is the phosphatase and tensin homologue deleted on chromosome 10 (PTEN). PTEN directly reverses the activity of PI3K by dephosphorylating PIP_3 into PIP_2 and therefore plays an important role as a negative controlling element of incoming signals. PIP_3 transduces activating

signals by binding to pleckstrin homology (PH) domains of proteins, thereby recruiting them to the cell membrane. One centrally important downstream mediator of the PI3K signalling cascade is the serine/threonine kinase Akt (murine thymoma viral oncogene homolog 1). Akt is recruited to the membrane via PIP_3 which is binding to its PH domain, followed by phosphorylation leading to its activation by the phosphoinositid-dependent kinase 1 (PDK1) at threonine 308 and at serine 473 by the mammalian target of rapamycin complex 2 (mTORC2). Activated Akt then mediates signals promoting cellular growth and survival and suppresses pro-apoptotic signals. Akt phosphorylates several intracellular proteins, including forkhead box O transcription factors (FoxO), the BCL2-associated agonist of cell death (BAD), and the glycogen synthase kinase 3 (GSK3), to promote cell cycle entry and cell survival (29). The proteins TSC1 (Hamartin) and TSC2 (Tuberin) form a complex that inhibits the activity of the small G-protein ras homologue enriched in brain (Rheb), which is necessary for mTOR complex 1 (mTORC1) activation. The Akt-mediated phosphorylation of TSC2 releases Rheb from its inhibited state. Rheb then accumulates in a GTP-bound state and can directly activate mTORC1, which phosphorylates the p70S6 kinase (S6K1) and the eukaryotic translation initiation factor 4E binding protein 1 (4EBP1), leading to increased protein translation (30) (Figure 2).

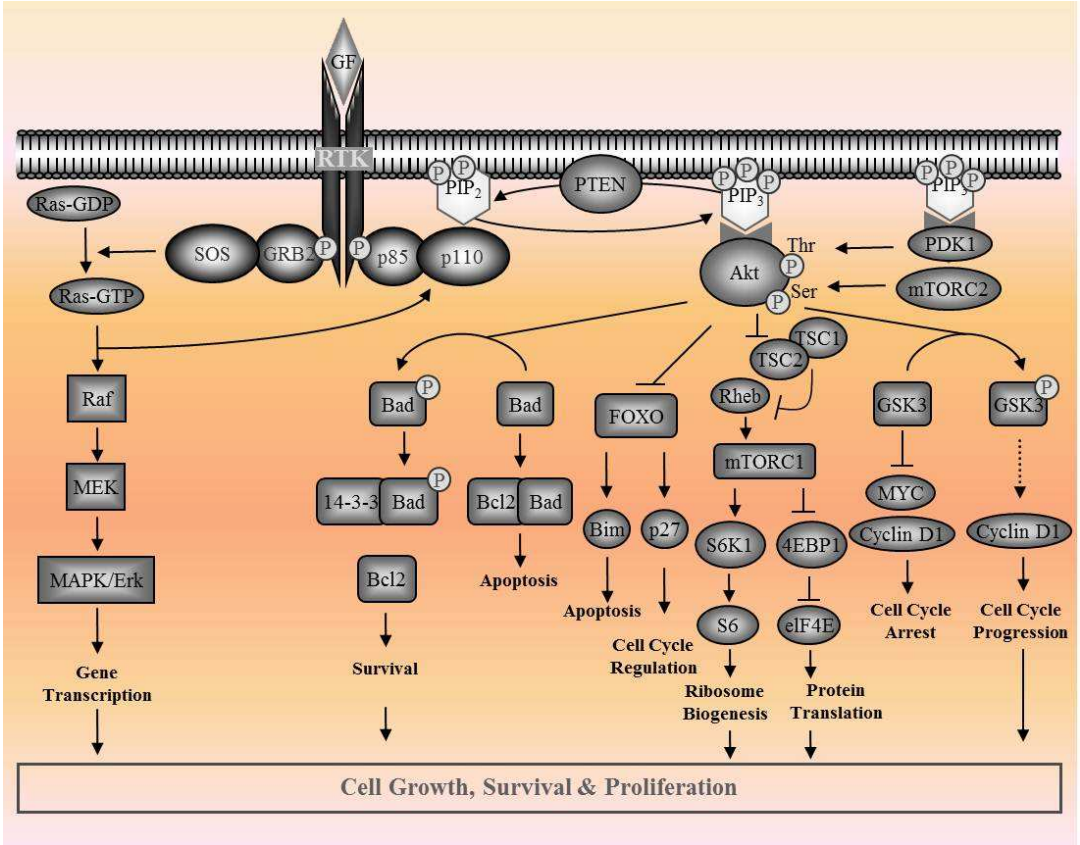

Figure 2 Scheme of the Class I PI3K Signalling Pathway Activation.

3.2 Cell Death Mechanisms

Death and degradation of cells is part of the normal self-renewal machinery in healthy eukaryotic organisms keeping a balance between continuous cycling of growth, proliferation, and cell death. Cytoplasmic organelles also constantly undergo reorganisation and destruction within the cells. Three main types of processes with the ability to finally lead to cell death have been identified, namely apoptosis, autophagy, and necrosis. Necrosis, even though now thought to be initiated and regulated under particular circumstances, has been traditionally described as an accidental type of cell death, whereas the so called programmed type of cell death, apoptosis, is an evolutionary highly conserved biochemical mechanism, protecting the organism from damaged cell accumulation, keeping homeostasis, and controlling cell fate. Autophagy, actually functional as a pro-survival mechanism, has a particular role in the biology of normal or cancer cells, playing a not yet fully understood Janus role between a cell survival and cell death mechanism. The regulation and involvement of apoptosis and autophagy in cellular biology and the contribution of their deregulation to key events in carcinogenesis will be summarized below.

Programmed Cell Death - Apoptosis

Apoptosis is one type of the programmed cell death (PCD) mechanisms characterized by series of biochemical events that lead to a variety of morphological changes, finally resulting in principal shrinkage of the cell and its nucleus, subsequently to cleavage of cytoskeletal proteins and collapse of subcellular components, as well as nuclear fragmentation, chromatin condensation, chromosomal DNA fragmentation, and the formation of plasma-membrane blebs (31). During animal tissue pattern formation PCD is required for the removal of unnecessary or excess cells. Apoptosis plays the main role in the regulation of tissue homeostasis and represents an important negative regulatory event protecting the organism from genome instability, therefore contributing to prevention from carcinogenesis, or in turn to death of cancer cells. Thus, deregulation in apoptotic processes has been described as one of the hallmarks of cancer (32). Mechanisms that confer increased resistance to apoptotic signals may occur at many different levels of apoptotic regulation, causing (acquired) resistance to anti-cancer treatments. This can be illustrated by the observation that more than 50% of neoplasms harbour defects in the apoptosis machinery. Overexpression of several pro-survival Bcl2 family proteins and mutations in the tumour-suppressor gene *TP53* are the best characterized among these abnormalities (33).

Apoptosis is divided into two different pathways, the extrinsic, so called death-receptor pathway and the intrinsic pathway, regulated by mitochondrial involvement.

Death Receptor Pathway. The extrinsic cell death pathway is activated when members of the tumour necrosis factor (TNF) super-family bind to “death receptors” localized at the cell membrane. Ligation of these receptors initiates the formation of the multiprotein death-inducing signalling complex (DISC) (34). DISC formation triggers the activation of Caspase-8, which then triggers either direct cleavage of

the downstream target Caspase-3, inducing apoptosis without involvement of mitochondria (35), or leads to apoptosis via a process involving mitochondrial cytochrome c release.

Mitochondrial Death Pathway. The interplay between pro-apoptotic and anti-apoptotic members of the Bcl2 family at the mitochondria regulates the execution of the intrinsic apoptotic pathway. Overwhelming cell damage caused by intracellular reactive oxygen species (ROS), DNA damage, or deprivation of growth factors is detected by intracellular sensors. Increased permeability of the mitochondrial membrane induced by apoptotic triggers results in the release of pro-apoptotic proteins (e.g. cytochrome c) into the cytosol. The subsequent formation of the apoptosome by recruitment of Apaf1 and pro-caspase 9 leads to the activation of caspase 9, which in turn activates caspases 3, 6, and 7, proteases that herald demolition of the cell by cleaving numerous substrate proteins and activating DNases (31, 33).

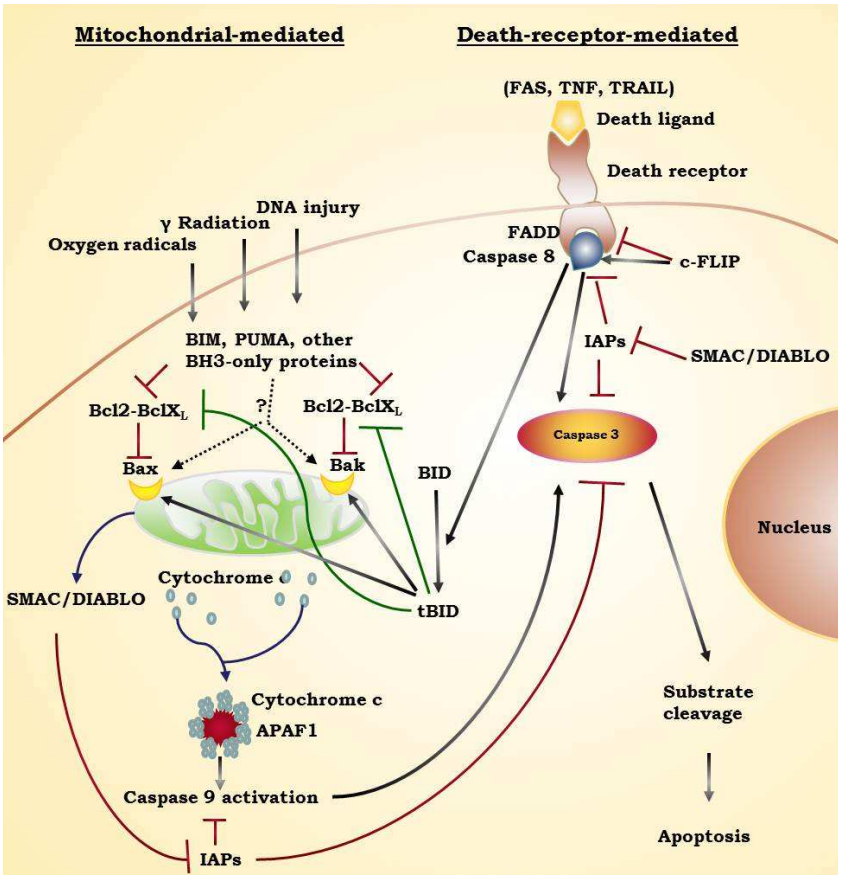

Figure 3 The intrinsic and the extrinsic apoptotic pathways.

BCL2 FAMILY MEMBERS AND THE REGULATION OF APOPTOSIS BY PI3K/AKT

The balance between pro-apoptotic and anti-apoptotic Bcl2 family members controls the mitochondrial apoptotic pathway. To date, 25 members of the Bcl2 family of proteins have been identified (36). These proteins are localized to the mitochondria, the smooth endoplasmic reticulum (37), and perinuclear membranes (38). Bcl2 family proteins are characterized by the presence of up to four relatively short sequence motifs, which are termed Bcl2 homology domains (BH) (36). In human,

Bcl2 family members can be divided into three subfamilies based on structural and functional features (39). The anti-apoptotic subfamily (Bcl2, $BclX_L$, BclW, Mcl1, Bfl1/A1, and BclB) suppress apoptosis and contain all four Bcl2 homology domains (designated BH1-4). Some pro-apoptotic proteins, such as BAX, BAK, and BOK, contain Bcl2 homology 1-3 domains and are termed ‘‘multidomain proteins’’, whereas other pro-apoptotic proteins termed as ‘‘BH3-only’’ proteins, such as BIM, BAD, and BID, contain only the BH3 domain (40). The molecular surface of the anti-apoptotic Bcl2 proteins possesses a hydrophobic groove, the BH3 binding cleft, capable of binding the BH3 domain of the pro-apoptotic proteins.

The complex interplay of pro-apoptotic BH3 domains and the BH3-binding groove of anti-apoptotic proteins regulates the pro-apoptotic activity of Bcl2 family members (41).

The anti-apoptotic Bcl2-family members are mainly localized in the mitochondrial outer membrane, but have been found as well in the membranes of the endoplasmatic reticulum (ER) and the nucleus. They stabilize the mitochondrial membrane and prevent cytochrome release and its subsequent binding to the apoptosis activating factor 1 (Apaf1). By contrast, the pro-apoptotic proteins of the Bcl2 family are usually localized in the cytosol or the cytoskeleton.

The onset of apoptosis and the mechanisms by which cells undergo cell death was shortly described above. Activation of PI3K and downstream activation of Akt play critical roles in keeping cells alive by blocking apoptotic pathways. PI3K/Akt signalling activation can block proteins triggering pro-apoptotic signals, and conversely promotes the expression of a subset of proteins mediating anti-apoptotic signals. In these regulatory processes, Akt plays a key role in the transduction of anti-apoptotic signals transmitted via PI3Ks (42, 43).

Akt has been demonstrated to phosphorylate the pro-apoptotic protein Bad, thereby mediating its inactivation through its binding to the cytosolic 14-3-3 proteins (44-47). Active Bad is able to capture Bcl2, and therefore to promote apoptosis upon freeing Bax from its inhibited state by Bcl2. Also Bax, the key regulator of the mitochondrial permeability leading to the release of cytochrome c into the cytoplasm, can be directly regulated by Akt via phosphorylation at Ser^{184} (48) and indirectly via the inhibition of GSK3 (49, 50), which phosphorylates Bax at Ser^{163} (51) leading to its activation. Furthermore, Akt suppresses the degradation of the anti-apoptotic Mcl1, which is promoted by GSK3 (52, 53).

Additionally, Akt regulates transcription factors, which are involved in the transcriptional regulation of apoptotic proteins. The forkhead transcription factors are negatively regulated by Akt (54-58). Their phosphorylation by Akt leads to sequestration and degradation in the cytoplasm (56). Active FoxO increases the expression of the Fas death receptor ligand FasL (59) and the pro-apoptotic BH3-only protein Bim (60). The latter was shown to promote apoptosis by binding to pro-survival Bcl2-family members, but has also been reported to directly mediate activation of Bax (61, 62).

Another transcription factor known to regulate genes involved in cell survival processes is NfκB (63). The expression of the pro-survival proteins Bcl2 and $BclX_L$ is known to be positively regulated by

NFκB (64-70), as well as several inhibitors of apoptosis (IAPs) (71). NfκB remains in the cytoplasm and is inactive when it is bound to its associated inhibitory molecule IκB (inhibitor of NFκB) (72). IκB is phosphorylated by a kinase complex consisting of the IκB kinases IKKα and IKKβ, leading to its ubiquitination and degradation, thereby releasing NFκB and allowing its translocation into the nucleus. The IKKα can be activated by multiple kinases, one of which is Akt (73, 74).

Furthermore, the tumour suppressor p53 is indirectly regulated by Akt. Upon irreversible DNA-damage p53 can regulate apoptosis by a combination of events, including up-regulation of pro-apoptotic proteins, such as Bim and PUMA. The activity of p53 can be attenuated by a regulatory protein called Mdm2 (murine double minute 2), which can translocate into the nucleus upon Akt phosphorylation and promote the ubiquitination of p53 (75).

All together, these observations illustrate the importance of PI3K signalling in apoptosis regulation processes and its importance as pro-survival pathway.

AUTOPHAGY - CELL SURVIVAL AND PROGRAMMED CELL DEATH MECHANISM

Autophagy was originally identified as a survival mechanism subsequent to starvation-induced cellular stress. It is a lysosomal degradation pathway for the breakdown of intracellular proteins and organelles and a highly regulated cellular process controlled by the evolutionary conserved autophagy-related (ATG) genes. In healthy cells, autophagy functions as a catabolic process by which cells recycle their own non-essential, redundant, or damaged organelles and macromolecular components. Beyond this homeostatic function, autophagy is a process by which the cells can adapt their metabolism to starvation or cellular stress. The functional relationship between autophagy and apoptosis is complex. Depending on the cellular context, autophagy may occur as a stress response to pro-apoptotic signals and thereby can interfere with the action of chemotherapeutic agents or radiotherapy, causing increased survival of the tumour cells. The mechanisms by which autophagy promotes cell survival are not restricted to its role in maintaining cellular energy homeostasis during starvation. Autophagy is also involved in removing damaged mitochondria and other organelles. In this context, autophagy can promote cell survival also during aging, infectious diseases and neurodegenerative processes. In fact, increasing evidence underlines the cyto-protective role of autophagy that allows to increase tumour cell survival under conditions of metabolic stress and hypoxia as well as to escape chemotherapy-induced cell death.

Autophagy is mediated by proteins named as autophagy-related (ATG) proteins (76) and can be divided into several stages: Induction, autophagosome nucleation, expansion and completion followed by lysosome fusion, degradation, and recycling (77). Approximately 30 ATG genes have been identified so far. Activation of the class III PI3K (Vps34) and Beclin1 (ATG6) are crucially required to recruit proteins and lipids for initiation of autophagosome formation mediated by ATG1-ATG13-ATG17, the modification and cleavage phosphatidylethanolamine(PE)-LC3-I to LC3-II by ATG4-ATG7-ATG3, as well as the transportation and elongation of the autophagosome by the ATG16-

ATg5-ATG12 complex (78, 79). Downstream of the class III PI3K, mTOR plays an important role as in the survival response initiated by withdrawal of growth factors or starvation and has been shown to act as a negative regulator of autophagy (27, 80).

However, there is also clear evidence for a role of autophagy in programmed cell death. As autophagy may often occur under conditions of stress, such as nutrient deprivation, finally causing cellular death, the concept of autophagy as a programmed cell death mechanism has been developing. This idea was mainly proposed after the observation that dying cells often display increased expression of autophagy markers, as well as morphological features of an autophagic phenotype. Accumulating evidence points to a complex interplay between the apoptotic and autophagic machinery, with autophagy playing an intermediate role finally resulting in apoptosis induction, or independently mediating cell death signals on its own followed by directly mediated autophagic cell death (81-83). However, the molecular mechanisms underlying the latter and its regulating processes have still to be investigated.

3.3 LUNG CANCER

Lung cancer is the leading cause of cancer-related mortality in the Western world and every year approximately 1.4 million people are diagnosed with lung cancer (84). Most commonly, lung cancer development is related to multiple genetic changes caused by exposure to carcinogens, for instance those found in tobacco smoke. Lung cancer can be divided into two main subtypes – non-small cell lung carcinoma (NSCLC) and small-cell lung carcinoma (SCLC). These subtypes differ not only in frequency of occurrence and prognosis, but also in biological behaviour, histological background, and characteristic genetic alterations.

Non-small cell lung cancer accounts for approximately 85% of all lung cancer cases, and is commonly related to tobacco smoking. In 9-15% of cases it is caused by exposure to other carcinogenic factors, such as polycyclic hydrocarbons, asbestos, or radon. Squamous cell carcinoma (SCC), adenocarcinoma (AC), and large cell lung carcinoma (LCLC) are the three most common types of NSCLC. The current treatment of NSCLC includes surgery, radiotherapy, and platinum-based chemotherapy. At the time of presentation at least 40% of patients are diagnosed with an advanced stage of disease (85), whereas less than 25% show early stage disease (stage I) (86). Surgery, with lobectomy or pneumonectomy being the most common surgical resections, remains the standard of care for patients with early stage NSCLC able to safely undergo surgery (86). The treatment of advanced NSCLC, which is mostly inoperable and therefore incurable, is aimed at controlling the disease to prolong life and sustain life quality, and commonly includes a combination of radio- and chemotherapy. An increasing progress in the optimisation of chemotherapeutic regimens in combination with targeted therapies against individual activated oncogenes has led to an improvement of NSCLC outcome, but the median 5-year survival rate nowadays is still only 15% (87).

3.4 SMALL CELL LUNG CANCER

HISTORY & CHARACTERISTICS

Small cell lung cancer accounts for approximately 15% of all lung cancer cases and is almost entirely (95%) related to tobacco smoking (88). It is characterized by rapid growth and early metastasis. Thus, surgical resection is rarely possible. Chemotherapy with etoposide- and platinum-based agents and in some cases also radiotherapy remain as the treatment options of choice (88-90). Although SCLC initially responds to therapy, most of the patients relapse. Due to metastasis and resistance to chemotherapy the clinical outcome is very poor and SCLC patients show an overall 5-year survival of less than 5% (89), with 90-95% of affected individuals dying of the disease within 5 years (88). However, the initial therapeutic response is followed by relapse and progressive development of chemotherapy resistance, and therefore the outcome is still very poor (91, 92). A slight decrease was observed in the incidence of SCLC cases over the last two decades, which could be explained by a decreasing number of smokers and changes in cigarette compositions, thus confirming the idea that SCLC is a highly preventable disease.

SCLC was first described in 1926 as lung cancer by Barnard as “oat-celled sarcomas of the mediastinum” (93). Together with the typical carcinoid (TC), atypical carcinoid (AC), and large cell neuroendocrine cancer (LCNEC), SCLC completes the subgroup of neuroendocrine tumours of the lung sharing common morphological, immunohistochemical and molecular characteristics. The histological criteria of SCLC include: the small size of the cells, limited cytoplasm, a nucleus with fine granulation, absent or faint nucleoli, a high level of mitosis, and frequent areas of necrosis (94).

DIAGNOSIS & STAGING

SCLC symptoms may occur due to local complications and distant metastases. At clinical presentation, SCLC patients often suffer from symptoms such as cough, chest pain, shortness of breath, and hoarseness of voice. Furthermore, indirect symptoms such as weight loss are commonly observed. The diagnostic and staging procedure for SCLC presently includes biopsies obtained from bronchoscopy, computed tomography (CT) scan of the chest and the abdomen, CT scan or magnetic resonance imaging (MRI) of the brain, radionuclide bone scans, and bone marrow aspiration. Also fluorodeoxyglucose (^{18}F) positron emission tomography (FDG-PET) and monoclonal antibody imaging are suggested to be potential initial staging tools with high accuracy compared to the currently used staging modalities used in SCLC (95, 96).

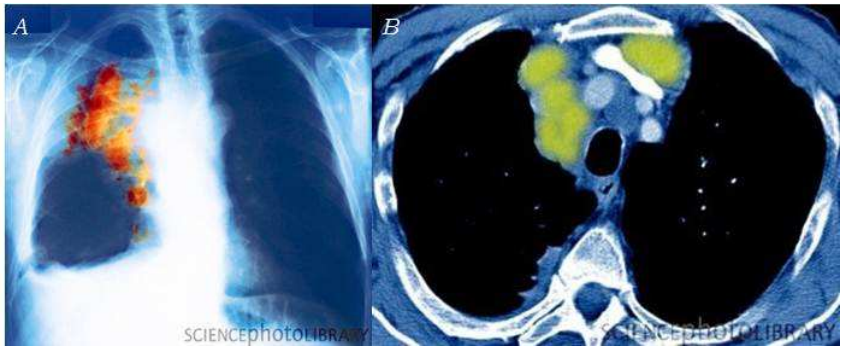

Figure 4 SCLC Imaging. A Coloured X-ray of the chest of a patient with small cell lung cancer (orange tumours, upper left, right lung). B Coloured CT scan in axial section through the chest showing cancerous lymph nodes (adenomegaly, yellow) as the primary site of SCLC. (Pictures adapted from www.sciencephoto.com)

Two different systems have been developed to analyse and characterize SCLC tumour stage.

i) According to the Veterans Administration Lung Group (VALSG) two-staged classification, first introduced in the 1950s, SCLC tumours are characterized as LD (limited disease) or ED (extensive disease). LD-SCLC represents about one third of the patients and is defined as disease confined to a localized tumour in one hemithorax, the mediastinum, and the supraclavicular lymph nodes, thus theoretically accessible within a "tolerable" RT portal. Among patients with limited stage disease, the median survival is about 23 months and the proportion surviving beyond 5 years is 12–17% (97). However at presentation, the majority of patients, (i. e. two thirds), show ED-staged SCLC, which is defined by locally advanced disease and/or metastatic spread. These patients have a median survival of 7–12 months and the proportion alive at 5 years is 2%.

ii) The classical TNM (tumour, nodule/regional lymph nodes, metastatic spread) staging system which was developed by Denoix in 1946 has remained as the traditional classification of primary NSCLC. As it requires accurate surgical lymph node sampling and at time of presentation two third of SCLC patients show locally advanced or metastatic disease and are rarely considered to be surgical candidates, the TNM staging system has not been routinely applied. The International Association for the Study of Lung Cancer (IASLC) has published the 7th edition of the TNM classification which seems more accurate in identifying patient subgroups. Limited disease should be based on this classification, corresponding to TxNxM0 patients and extensive disease corresponds to TxNxM1a and TxNxM1b patients (96, 98).

TREATMENT & PROGNOSIS

SCLC prognosis may be in part determined by clinical, radiological or biological factors assessed before any treatment. The most important prognostic factor is limited disease spread. SCLC patients with LD-staged SCLC, good performance status, female gender, age <60 years, normal LDH (lactatdehydrogenase), and stage I disease are associated with more favourable prognosis. In terms of prognosis, poor performance status, extensive disease, and weight loss at the time of diagnosis are important adverse indicators for ED-SCLC (96, 99).

Surgery as first-line treatment for LD-staged SCLC still remains controversial. Initially it was the treatment of choice for all lung cancers. But 30 years ago, it was mostly abandoned after a randomized clinical trial in which radiotherapy showed an advantage upon surgery on the 5 year survival in SCLC patients with limited disease. Nowadays, the most favoured therapeutic approaches for LD are combination therapies consisting of chemotherapy involving cisplatin or carboplatin with etoposide and thoracic radiation (chemoradiotherapy). Additionally, prophylactic cranial irradiation (PCI) is successfully applied to prevent the high risk of brain metastases. There is also the opinion rising that surgery should again play a role as primary treatment followed by chemotherapy for T1N0 and possibly in T2N0 or included as adjuvant treatment in an approach of multimodality treatments (96, 99, 100).

ED-staged SCLC is usually treated with a combination of chemotherapies (cisplatin + etoposide = EP) as first-line settings. Different first-line settings were applied and studied and compared to EP treatment in clinical trials, e. g. cyclophosphamide + doxorubicin + vincristine (CAV), irinotecan + cisplatin (IP), or EP + paclitaxel. Overall, only IP treatment resulted in similar effects compared to EP, but increased the incidence of gastrointestinal toxicities, and EP continues to be the treatment of choice for ED-staged SCLC patients. Radiotherapy is applied as palliative care and prevention of brain and bone metastasis, but does not prolong survival (96, 99, 100).

Although SCLC is a highly chemoresponsive disease, the development of treatment resistance remains a problem and the majority of patients (80% LD patients, almost all ED patients) relapse. Second-line treatment in refractory or resistant SCLC includes, beside the re-treatment with the original regimen, also topotecan regimens, based on the performance status (PS) of the patient. Even though improvements in chemotherapy regimens have been made and a better understanding of SCLC biology could be developed, the survival of SCLC patients has not greatly improved in the last 25 years. The development of resistance to chemotherapy and metastasis are commonly recognized as important causes of poor clinical outcome in SCLC (96, 99, 100).

MOLECULAR PATHOGENESIS & GENETIC ALTERATIONS

The molecular mechanisms responsible for lung cancer formation have been extensively studied. A striking event in the development of cancer is the escape of atypical cells from the normal growth control and their transformation into a malignant and invasive phenotype. Numerous genetic and molecular alterations have been reported to be associated with the development of SCLC, including autocrine signalling loops, oncogene activation and loss of tumour-suppressor genes (88). These genetic and molecular aberrations result in a lack of response to negative growth regulatory signals and the continuous presence of positive signals that regulate growth, motility, and invasion.

The exact cellular origin of SCLC remains unclear (101). Early work suggested SCLC may arise in the bronchial mucosa with displaying some epithelial characteristics. Furthermore, stem cell populations of the lung, such as certain Clara cell types associated with neuroendocrine bodies, were identified to

be possibly involved into SCLC tumourigenesis. A mouse model was developed in which SCLC tumours were initiated by the deletion of *RB1* and *TP53* tumour suppressor genes in the lung epithelium of adult mice (102). Additionally, it was shown by Parks *et al* that in lung epithelium with deletion of *TP53* and *RB*, SCLCs arose where neuroendocrine cells were located and that early lesions were mostly composed of proliferating neuroendocrine cells, whereas mice, in which *RB* and *TP53* were deleted in non-neuroendocrine cells did not develop SCLC (103). Just recently, Sutherland *et al.* demonstrated that loss of *TP53* and *RB* could transform pulmonary neuroendocrine cells (NE) but not Clara cells and concluded that NE cells rather than stem cells might be the predominant cellular origin and the target of SCLC (104). SCLC tumourigenesis is also thought to be related to deregulations in developmental pathways like Hedgehog-, Notch, or Wnt-signalling, which are known play a role in developmental processes of the lung (105).

Multiple neuropeptides and polypeptides promote the growth of SCLC cells in an autocrine/paracrine fashion. Amongst the neuropeptides, gastrin-releasing peptide and neuromedin B have been shown to be expressed in SCLC, along with their specific receptors (88, 106).

As seen in many solid tumours, loss of genomic stability does also occur in SCLC. Several chromosomal abnormalities have been observed to occur frequently in SCLC. The majority of SCLCs show deletions affecting multiple chromosomal sites including losses at 3p, 5q, 13q, and 17p, carrying the genetic information for tumour suppressor genes, such as *RB* and *TP53*. In contrast, a large number of SCLC tumours harbour gains of 1p, 2p, 3q, 5p, 8q, and 19p, regions known to encode for oncogenes, such as *KRAS* and *MYC*. A more aggressive phenotype was demonstrated in SCLC cells bearing coexistent deletion of 18q and amplifications of 1p, 2p, and 3q, underlining once more the importance of the genetic and epigenetic disorders of a disease. Allele loss of chromosome 3p occurs with a frequency higher than 90% in SCLC and is believed to be an early event in lung cancer development. Several genes located on the regions 3p21.3, 3p12, 3p14.2, and 3p24 show tumour suppressor activity and lose their expression via epigenetic mechanisms. The fragile histidine triad gene (*FHIT*) encodes the enzyme diadenosine triphosphate hydrolase, which is thought to have an indirect role in apoptosis and cell-cycle control. The RAS effector homologue (*RASSF1*) encodes a microtubule-binding protein. *RASSF1* interacts with microtubules, causing their stabilisation and thus inducing G1 and G2/M arrest. The loss of *RASSF1* function allows tumour cells to grow more rapidly. The third and fourth potential tumour-suppressors are the retinoic acid receptors β, through which retinoids trigger the induction of apoptosis and *FUS1*, found at 3p21.3, which induces cell cycle arrest and apoptosis but loses expression of its protein in 100% of SCLCs (88, 105, 107).

TUMOUR SUPPRESSOR GENES

TP53. Located on chromosome 17p13.1 the tumour suppressor gene *TP53* is also called “the gatekeeper of the cell”. By regulating cell survival and cell damage response signalling pathways its protein product is aimed to protect the cell against genetic disorders and instability. The transcription

factor acts as a negative regulator of cell proliferation via targeting genes involved in the control of cell cycle regulation, apoptosis, and DNA repair. *TP53* is mutated in 50% of human cancers and also plays an important role in development of SCLC (108). Inactivating mutations (missense mutations in the DNA binding domain and homozygous deletions) of the *TP53* gene could be observed in approximately 90% of SCLCs (109) and are known to be partially caused by carcinogens found in tobacco smoke (110). Additionally, 40-70% of SCLC tumours show an abnormal expression of the p53 protein (108). Mutated *TP53* was found to be a potential target in cancer immunotherapy due to its prolonged half-life and increased expression in cancer cells. In SCLC, a vaccine composed of dendritic cells transduced with a human recombinant adenovirus containing wildtype p53 (DC-Ad-p53) resulted in a partial response in a minority of patients and is currently applied in several clinical trials involving ED-staged SCLC patients (105, 111).

Retinoblastoma. The retinoblastoma gene *RB1* located on chromosome 13q14.1-q14.2 encodes for a nuclear protein involved in mediating the G1/S transition in cell cycle progression. Hypophosphorylation of Rb causes cell cycle arrest and growth suppression by controlling transcription factors of the E2F family necessary for cell cycle progression. Phosphorylation of Rb by cyclin D1/CDK4 reactivates E2F transcription factors, followed by transition to S-phase and inhibition of pro-apoptotic proteins. 90% of SCLCs show abnormalities in RB, with either complete absence of protein or expression of a mutated form (112, 113). Together with loss of p53 the loss of Rb is suggested to be a major feature in SCLC development.

RECEPTOR TYROSINE KINASES

Receptor tyrosine kinases are involved in cellular proliferation, survival, and migration, mediating positive growth signals via activation of downstream signalling molecules. In SCLC, deregulation of RTK signalling often occurs due to overexpression of RTKs and their respective growth factors, leading to multiple autocrine/paracrine signalling loops, including stem cell factor (SCF)/c-Kit, insulin-like growth factor-I (IGF-I)/IGF-IR, vascular endothelial growth factor (VEGF)/VEGFR, fibroblast growth factor (FGF)/FGFR, and hepatocyte growth factor (HGF)/c-Met, which all lead to the activation of PI3K/Akt signalling and promote cell growth, survival, and chemotherapy resistance. Mutations are observed to a lesser extent pointing to the importance of the translational regulation of the RTK signalling pathway in SCLC.

c-Kit. c-Kit is a member of the PDGF/c-Kit receptor tyrosine kinase family. Upon binding of its ligand stem cell factor (SCF) activation of the JAK-STAT, PI3K and MAP kinase pathways are initiated, promoting cell growth and differentiation (114). The co-expression of c-Kit and SCF is rare in human cancers, but was shown to be a common event in SCLC cell lines and tumours and was the first autocrine signalling system described in SCLC (115-117). However, c-Kit expression in SCLC patient tumour tissue was found to range from approximately 20-90% and also the association of its expression with patient survival resulted in controversial observations, ranging from no relevance or

poor prognosis to prolonged survival (118). In preclinical studies the c-Kit inhibitor Gleevec (Imatinib, STI571) was partially able to inhibit the SCF-induced activation of Akt and Erk in a cell-dependent manner (119-123), whereas in clinical trials Gleevec failed to display anti-tumour activity in SCLC (124-128).

IGF-IR. Expression of the IGF-IR and its main ligand IGF-I have been observed in SCLC cell lines and in cells derived from SCLC patients, suggesting an autocrine signalling loop by this system (128-132). Additionally, increased gene copy number and occasional occurrence of gene amplification have been identified. Indeed, involvement of the IGF-IR pathway in the development and growth of SCLC has been reported and is known to function via activation of the PI3K/Akt pathway stimulating SCLC tumour growth, survival, and resistance to chemotherapy. Preclinical studies employing IGF-IR-inhibitors (e.g. NVP-ADW742) or neutralizing antibodies (e.g. A12) resulted in growth inhibition and apoptosis in SCLC cells. Furthermore, inhibition of IGF-IR signalling synergistically enhanced the sensitivity of SCLC to etoposide and carboplatin. This enhancement in sensitivity to chemotherapy tightly correlated with inhibition of PI3K-Akt activation (121, 133, 134).

c-Met. The receptor tyrosine kinase c-Met is activated by its ligand hepatocyte growth factor (HGF) triggering positive growth signals via the PI3K/Akt signalling cascade in SCLC and was shown to be implicated in the development of an aggressive and invasive metastatic SCLC tumour phenotype (135-137). Overexpression and amplification of c-Met have been demonstrated in SCLC and indeed, high levels of HGF were associated with a poor prognosis. A mutational analysis of c-Met in SCLC identified several alterations, upon which mutations in the juxtamembrane domain were shown to regulate cell proliferation, cell morphology and adhesion, as well as causing enhanced cell motility and migration (138).

VEGFR. The family of the vascular endothelial growth factors and their corresponding receptor tyrosine kinases comprises numerous VEGFs and three distinct receptors. The VEGF signalling pathway leads to increased proliferation, migration, and invasion of endothelial cells, thus making them key players in mediating tumour angiogenesis. All three VEGFRs are expressed in SCLC (139). Autocrine signalling loops of VEGF/VEGFR mediating proliferative and migration signals have been shown in SCLC cells and a significant correlation of VEGF expression and vessel density could be demonstrated in SCLC xenografts (140). High levels of VEGF were reported to correlate with tumour stage, disease progression, and resistance to chemotherapy, thus leading to a poorer outcome in SCLC patients (141-144). Whereas an association of microvessel density and tissue VEGF expression could be correlated to a poorer prognosis in NSCLC patients, these observations could not be confirmed in SCLC, even though higher vascularization was observed in SCLC compared to NSCLC tumours (145).

FGFR. The fibroblast growth factor receptor family comprises four different isoforms. These RTKs are activated upon binding of their ligand fibroblast growth factors (FGFs) and interact with numerous signalling molecules, triggering signals via the Ras/Raf/MEK/Erk1,2 and PI3K/Akt pathways.

Elevated levels of FGF-2 have been found in serum of SCLC patients correlating with poor outcome and increased angiogenesis (146). Additionally, FGF-2 was shown to rescue SCLC cells from chemotherapy-induced apoptosis by an increased expression of anti-apoptotic proteins, such as $BclX_L$ and XIAP (147-150). The selective FGFR inhibitor PD173074 was shown to block SCLC proliferation and clonogenic growth and to prevent FGF-2-induced chemoresistance *in vitro*, and displayed not only increased apoptosis, but also decreased intratumoural proliferation *in vivo* (151).

PROTEINS OF THE BCL2 FAMILY IN SCLC

Alterations in the expression of Bcl2 are involved in the pathogenesis of human malignancies. Anti-apoptotic Bcl2 proteins are typically overexpressed in many cancers and associated with drug-sensitivity. Even though no mutations or amplifications in the gene encoding for the Bcl2 protein have been identified until now in SCLC, Bcl2 was found to be up-regulated in 70-90% of SCLC tumours (152-155), which partially could explain their apoptotic dysfunction and resistance to programmed cell death upon treatment with chemo- and radiotherapy. Bcl2 has been shown to be associated with increased cell survival in *in vitro* models of SCLC (149, 156, 157). Upregulation of Bcl2 inhibited apoptosis induced by cisplatin, doxorubicin and etoposide and furthermore was required for the maintenance of drug resistance (157-161). Conversely, inhibition of Bcl2 resulted in anti-tumour activity in SCLC cell lines and xenografts (162-164). However, an *in vivo* confirmation of an important role of Bcl2 in patients with SCLC is rare. No relationship between Bcl2 expression levels and patient survival has been demonstrated in SCLC (164). A study employing TMA meta-analysis of different former studies, associated a slight survival advantage with low Bcl2 expression, but did not show a clear relationship between Bcl2 expression and patient survival (165). Despite this, several trials of treatments targeting Bcl2 in SCLC (e.g. the Bcl2 inhibitors ABT737 and obatoclax) are in progress and have reached clinical stage (166-173).

PI3K SIGNALLING IN SMALL CELL LUNG CANCER

Numerous genetic and molecular alterations have been reported to be associated with the development of SCLC, including autocrine signalling loops, oncogene activation and loss of tumour-suppressor genes (88). These genetic and molecular aberrations result in a lack of response to negative growth regulatory signals and the continuous presence of positive signals that regulate growth, motility, and invasion.

Mutation in the *PIK3CA* gene, known as one of the most common genetic alterations present in human cancers, were found in primary SCLC (13%) and in SCLC cell lines (23%) (174). Another study performed in 2008 failed to detect any mutations in the exons 9 and 20 of *PIK3CA*, but reported *PIK3CA* copy number gains (4.7%), which correlated with higher expression of activated Akt in SCLC cell lines (175). Chromosome 3q26-ter amplification including the *PIK3CA* gene locus was also shown in 67% of SCLC tumour samples (176). A recently published study showed that genes

encoding components of the RTK/PI3K/mTOR axis and apoptosis-regulating proteins harbour high frequencies of copy number alterations in both SCLC tumours and SCLC cell lines (177). Copy number gains could be identified in the *PIK3CA* gene in 76% of SCLC tumours and in 54% of the SCLC cell lines. The *AKT1* gene was amplified in 64% of tumours and 39% of cell lines. In addition, the gene *FRAP1* encoding the downstream target mTOR was shown to be amplified in more than 50% of SCLC tumours. In view of the loss of *PTEN* in 76% of SCLC tumours, the members of the PI3K/Akt pathway were suggested to be potential drug targets of SCLC in this study (177). PTEN plays an important role as a negative regulator of the PI3K/Akt signalling pathway by acting as direct antagonist of PI3K and is mutated (~15%), or can be deleted in SCLC (178-180). Constitutively activated PI3K was found in SCLC cell lines and shown to promote growth and anchorage-independence due to high levels of basal Akt and $p70^{s6k}$ activity in the late 1990s (181). Differential over-expression of several PI3K isoforms and their contribution to Akt activation were demonstrated in different SCLC cell lines. In particular, cells over-expressing the PI3K subunit p110α showed enhanced Akt activation after growth factor stimulation. PTEN down-regulation could not be observed as a plausible reason for the higher Akt activity (182). Additionally, in tumour tissue samples from SCLC patients high levels of phosphorylated Akt (~50-70%) were detected by immunohistochemistry, supporting the involvement of the activated pathway in disease progression (176, 183). Other studies related integrin-induced activation of PI3K/Akt signalling and adhesion on extra cellular matrix (ECM) with resistance to various therapies and protection from apoptosis (123, 184). Adherent-growing SCLC cells, which are those thought to initially survive chemotherapy, were reported to activate Akt and thus to increase chemo- and radiotherapy resistance by mechanisms involving over-expression of anti-apoptotic proteins (185). One possible mechanism by which SCLC cells can escape the effects of cytotoxic drugs was discovered in experiments elucidating SCLC responses to cisplatin, a DNA-damaging agent. Surprisingly, treatment with cisplatin up-regulated Akt activation and contributed to the expression of pro-survival proteins in SCLC cells (186).

Several autocrine loops have been described in SCLC cells, including stem cell factor (SCF)/c-Kit, insulin-like growth factor-I (IGF-I)/IGF-IR, (VEGF)/VEGFR and hepatocyte growth factor (HGF)/c-Met, which all lead to the activation of PI3K/Akt signalling and promote cell growth, survival, and chemotherapy resistance. Direct inhibition of the PI3K/Akt pathway with the PI3K-specific inhibitor LY294002 attenuated these effects on cell growth, led to apoptosis, and enhanced the apoptotic effects of chemotherapeutic agents, such as cisplatin or etoposide (123, 187). The same effect was observed after inhibition of Akt by expression of a dominant-negative mutant of this protein in SCLC cells (187). Inhibition of Akt with the small-molecule inhibitor Tricribine (known as AKT/Protein kinase B signalling Inhibitor2) resulted in significantly affected growth and colony formation and pointed out a higher sensitivity of *PIK3CA*-mutated SCLC cells compared to *PIK3CA* wild-type cells (174). Moreover, the use of specific RTK inhibitors for c-Met, c-Kit, IGF-IR (121, 133, 188, 189), alkaloids

(naltrindole; opioid receptor antagonist) (190) or antibodies (134) were reported to impair SCLC cell survival in a PI3K/Akt-dependent manner.

Targeting PI3K signalling with pharmacological inhibitors has become an important experimental therapeutic approach. Targeting the RTK/PI3K/Akt cell survival axis and its downstream mediators with small molecule inhibitors (tyrosine kinase inhibitors, TKIs) is, beside treating cancer with classical chemotherapeutical agents, one of the most prominent approaches used in cancer therapy and has been reviewed in the past for various human cancers. The use of specific TKIs has led to a progress in cancer treatment options, especially in cancer types carrying a particular oncogene addiction, which leads to dependency on the activity of one particular receptor tyrosine kinase. In lung cancer, the knowledge about molecular alterations has predicted the use of TKIs, which are nowadays used in the clinics. However, the recurrence and metastasis of tumours, which are associated with a switch in the oncogene addiction and changing signalling pathway activation, followed by the development of chemoresistance have raised the awareness about other pathways and/or multi-point intervention to target different signalling nodes in parallel. Compared to the development and the use of TKIs in the clinics, the development of PI3K inhibitors appears to be still in an early clinical phase, but is rapidly processing. Looking at current or soon initiating clinical trials in various cancer types, it is clear that PI3K inhibitors have arrived to the clinical stage, but clinical data confirming pre-clinical results in SCLC are still awaited.

TOBACCO SMOKING AND PI3K SIGNALLING ACTIVATION

Small cell lung cancer development is almost entirely related to smoking, thus, raising the question whether components of tobacco smoke are involved and which pathways are activated during the carcinogenic processes. As a traditional model of tobacco-related tumourigenesis the theory has been established that tobacco components promote carcinogenesis through multistep processes which lead to the accumulation of mutations in key genes, such as *TP53*, *RB* or *KRAS*. A genomic study of a SCLC cell line (NCI-H209), derived from a bone marrow metastasis before chemotherapy, associates the carcinogens found in tobacco smoke with genetic alterations ("tobacco smoke-related signature") in the genome of SCLC tumour cells. These genetic aberrations may cause tumour formation through circumvention of cell damage-induced cell death. Additionally, nicotine and the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NKK), both important components of tobacco smoke, were associated with early biochemical events occurring in cells exposed to tobacco smoke (184, 191, 192). In an *in vitro* model of normal primary human airway epithelial cells ((i) normal human bronchial epithelial cells (NHBEs), precursor of SCC; ii) small airway epithelial cells (SAECs), precursor of AC)), activation of the PI3K/Akt pathway was demonstrated to be an early event promoting cellular survival due to exposure to nicotine and NKK, suggesting a molecular mechanism to overcome cell damage-induced apoptosis involved in lung cancer development (184). Consistent with these results, another study identified PI3K pathway activation in normal and

premalignant bronchial airway epithelial cells of smokers with airway lesions, thus supporting the hypothesis that PI3K signalling activity is induced before the development of lung cancer (193). Nicotine was also shown to increase cell proliferation and survival accompanied by activation of the PI3K/Akt/mTOR pathway in NSCLC and SCLC cells (191, 192), and was associated with NFκB-dependent resistance to chemotherapeutic drugs (192). Expression of nicotinic acetylcholine receptors (nAChRs), which respond to nicotine and NKK and activate Akt through PI3K, was found in normal and in lung cancer cells (184, 191, 192). Another group reported that nicotine increased the expression of peroxisome proliferator-activated receptors (PPARβ/δ), which belong to the nuclear hormone receptor superfamily of ligand-dependent transcription factors, in a nAChR-mediated PI3K/Akt/mTOR-dependent manner, and thereby promoted NSCLC cell growth (194). Furthermore, PI3K/Akt activation in response to nicotine exposure led to phosphorylation of Bax through Akt, shortening its half-life and abrogating the pro-apoptotic activity of Bax. These events resulted in the promotion of cell survival (195). Bad, another pro-apoptotic member of the Bcl-2 family, was shown to undergo multi-site phosphorylation involving nicotine-induced activation of the MAPKs Erk1/2, PI3K/Akt, and protein kinase A (PKA), leading to suppression of apoptosis (196). Both studies could show that inhibition of the PI3K/Akt pathway abrogated nicotine-triggered anti-apoptotic signals and blocked lung cancer cell growth, suggesting that PI3K/Akt activation is an important step in nicotine-induced cancer cell survival (195, 196).

3.5 NEUROBLASTOMA

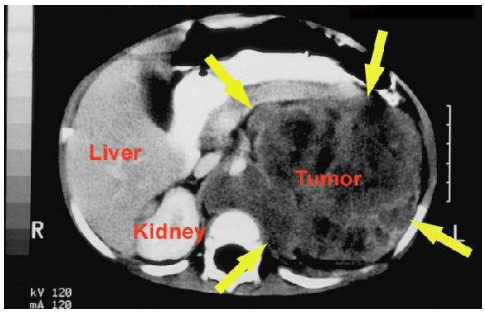

Figure 5 NB Imaging. The tumour is indicated by arrows.

The embryonal tumour neuroblastoma is the most common extracranial solid malignancy in childhood, causing around 15% of all childhood cancer related mortality (197). Embryonal tumours (ET) represent an important group of infant malignancies arising from different tissues of fetal origin. They are highly malignant and characterized by poorly differentiated neuroepithelial cells with the potential capacity for divergent differentiation. As a cancer of developing tissue, NB most commonly occurs in children before the age of 1 year with a median age of diagnosis being 17 month (198, 199). Its cell of origin is thought to be a developing precursor cell from the neural-crest (200), thus the tumour is typically arising in tissues of the sympathetic nervous system, such as the adrenal gland in 40% of the cases. Other localization sites of the primary lesions are common along the chain of the sympathetic nervous system (neck, chest, abdomen, or pelvis) (201). The symptoms experienced at clinical presentation differ depending on the stage of the disease and tumour localization. Approximately 50-60% of all patients show metastatic disease at time of diagnosis. The clinical behaviour of NB shows a

remarkable heterogeneity varying from frequently observed spontaneous and complete regression in very young patients with localized tumours to metastatic and progressive disease with resistance to multimodality treatments, ultimately leading to death (198, 202, 203). Even though advances in understanding the biology of neuroblastoma has been made, leading to an improved outcome over the last few decades, this improvement has mainly benefited patients with the more benign form of NB. The overall survival rate for high-risk NB patients is still less than 40% (204, 205).

STAGING & TREATMENT

The INSS (International Neuroblastoma Staging System) has identified distinct prognostic stages (1, 2A, 2B, 3, 4, 4S) (206). Based on the INSS stage, tumour biology, and age group, patients can be assigned to low-, intermediate-, and high-risk disease.

Initially, all patients with suspected NB undergo surgery to verify the histology and obtain material for molecular marker analysis. The exception to this rule are small infants less than 3 month of age in good clinical condition, which may stay under observation without surgery because of high rates of spontaneous regression. The types of treatments applied to NB patients is the classical oncological triad consisting of surgery, combined chemotherapy (cisplatin or etoposide with vincristine, doxorubicin, cyclophosphamide, or topotecan), and radiation.

Low-risk patients include those younger than one year of age at the time of diagnosis with low grade tumours lacking *MYCN* amplification (207). Around 45% of cases are assigned as low-risk patients requiring only surgery and/or observation of the disease (208, 209).

Intermediate risk patients are generally older than one year of age at the time of diagnosis with intermediate or high grade tumours. While copy numbers of *MYCN* are typically normal, other genetic alterations, such as 11q loss or 17q gain are often observed. The intermediate-risk group undergoes chemotherapy to reach tumour shrinkage prior to surgical resection. In the case of non-resectability, patients receive radiation therapy and retinoic acid as maintenance treatment (210, 211).

High-risk patients account for around 40% of all cases and are characterized by a highly aggressive type of NB, which is known to be difficult to cure. They are usually older than one year of age at the time of diagnosis and have intermediate- or high-grade tumours. These tumours harbour *MYCN* amplification and a number of other genetic alterations associated with unfavourable outcome. Therefore, the treatments applied are multimodality regimens, currently comprising high-dose chemotherapy, surgery, and radiation (212, 213). In the case of relapse and acquired drug resistance additional radioiodine labelled metaiodobenzylguanidine (I-131 MIBG) therapy with high dose chemotherapy is followed by rapid autologous stem cell rescue and retinoic acid maintenance treatment (205, 214).

The overall survival rate of all NB patients is approximately 65%, including an excellent prognosis (98%) of very young patients with localized disease, while only about 35% of high-risk NB patients survive.

GENETIC ALTERATIONS

NB tumours are formed as a result of genetic mutation and/or changes in epigenetic factors responsible for the right programming of the neural crest cells. Several genes have been discovered to drive the correct differentiation of the NC cells and are dysregulated in NB. While most NBs arise sporadically, a small number, approximately 1%, show familial inheritance (215, 216). Several characteristic genetic alterations with prognostic value have been discovered and associated with different patient subgroups. Genome-wide studies of NB tumours of different stages has been employed to identify genes useful to predict predisposition for NB, clinical outcome and understanding the biology of tumour progression (217, 218).

Genetic alteration in the *MYCN* gene, localized at 2p23-24, was one of the first aberrations discovered in NB and its expression level is used as molecular parameter to predict clinical prognosis in NB. MYCN is a member of the MYC family of basic helix-loop-helix transcription factors which control cell cycle progression, proliferation, cellular differentiation, DNA damage response, and apoptosis. Approximately 20% of NB tumours display amplification of *MYCN*. High levels of MYCN expression correlate with an aggressive tumour behaviour and poor clinical outcome (210). This is illustrated by the observation that over 40% of metastatic NB display *MYCN* amplification, whereas this rate is less than 10% for localized tumours (219).

Unfavourable tumours are further characterized by LOH deletions of 1p or 11q, as their appearance is more frequent in intermediate to high-risk tumours. LOH of 1p is often paralleled with *MYCN* amplification (220, 221). The allelic loss of 11q is rarely associated with *MYCN* amplification but occurs with other prognostic features, such as advanced disease and older age (222, 223). The frequent gain of 17q, where possible oncogenes such as BIRC5 (survivin), NME1 and PPMID are located, is also applied as prognostic factor in NB. Whole chromosomal gain can be associated with low-grade disease, whereas segmental gain is observed in high-grade tumours (224-227).

Additionally, tumours with whole chromosome gains or losses are associated with lower risk disease and favourable outcomes, whereas partial chromosomal aberrations are associated with high-risk disease and worse outcomes (198, 228). Thus, patient risk stratification due to the analysis of total DNA content (ploidy) is frequently used and considered to be a prognostic value (228).

Loss-of-function mutations in the gene paired-like homeobox 2B (*PHOX2B)* frequently occur in hereditary NB, leading to abnormalities in the neural crest (NC) derived tissue (229, 230). The *PHOX2B* gene encodes a transcription factor, essential for the regulation of neurogenesis (229, 230). Furthermore, single nucleotide polymorphism (SNP) variations within the genes *FLJ22536* at 6p22.3 and *BARD1* (BRCA1-associated ring domain 1) have been discovered to be enriched in NB patients (231, 232).

The anaplastic lymphoma kinase (*ALK)* gene encodes a receptor tyrosine kinase, which is involved in proliferation and differentiation pathways via activation of several signalling pathways in the nervous system. Constitutively activated ALK, based on activating mutations in the kinase domain of the *ALK*

oncogene, accounts for most cases of familial recurrent NB (233). Additionally, in somatically acquired NB the incidence of *ALK* mutation is still 5-15%, suggesting its importance for NB development (234). It has been reported that amplification of the *ALK* gene only occurs in *MYCN*-amplified primary NBs, but the mechanisms how the MYCN overexpression cooperates with the *ALK* amplification still remains elusive (233, 235). Recently, a transgenic zebrafish model has been generated in which overexpression of human MYCN in the peripheral sympathetic nervous system (PSNS) induces a tumour which closely resembles human NB (236). In this model, *MYCN* overexpression was required for NB development, whereas the expression of *ALK* was not sufficient, even though it has been demonstrated by Mosse *et al* that germline mutations of *ALK* can act as an initiating event in NB tumours with or without *MYCN* amplification (233). Co-expression of *MYCN* and an activated *ALK* enhanced the tumour onset and penetrance by developing a potent anti-apoptotic phenotype (236).

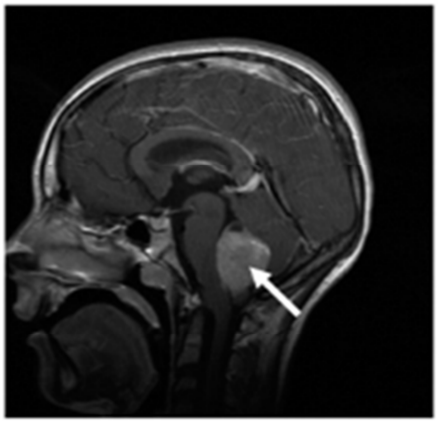

Figure 6 MB Imaging. The tumour is indicated by arrow.

3.6 MEDULLOBLASTOMA

Medulloblastoma is an embryonal tumour of the cerebellum with neuroepithelial background and the most common malignant brain tumour of childhood (237), accounting for approximately 20% of all CNS tumours. Embryonal tumours (ET) represent an important group of infant malignancies arising from different tissues of fetal origin. Among ET, the tumours of the central nervous system (CNS) account for approximately 20% of all childhood cancers, ranking second only to leukemias (238). They are highly malignant and characterized by poorly differentiated neuroepithelial cells with the potential capacity for divergent differentiation (239). MB is a highly invasive tumour arising in the posterior fossa, where it is thought to originate from several pluripotent precursor cells of the ventricular zone. Due to its malignant phenotype MB is considered as a grade IV tumour by the WHO (World Health Organization). Medulloblastoma patients can be subdivided into high-risk and standard-risk patients based on the clinical criteria and tumour histology. MB shows two peaks of incidence. One is at the age between 3 and 4 years, the second between 8 and 9 years of age, with a higher incidence in boys than in girls (240). Although MB can also occur in adults, it accounts for less than 1% of the adult CNS tumours (241). MB is characterized by an aggressive clinical behaviour and a high-risk of leptomeningeal dissemination (242, 243). Clinical symptoms of MB which are frequently present in children are ataxia, hydrocephalus, and increased intracranial pressure (ICP), paralleled by non-specific symptoms, such as headache, vomiting, and lethargy. The multimodality treatments consisting of surgery, radiotherapy, and chemotherapy were able to improve the overall survival rate in the past decades. The combination of chemotherapy and radiotherapy has improved the

5-year overall survival rate to 55-76% in high-risk patients and 70-80% in standard-risk patients. Even though approximately two thirds of patients without disseminated disease can be cured by applying currently available therapies, one third of the patients still remain incurable.

GENETIC ALTERATIONS IN MEDULLOBLASTOMA

The most frequently altered signalling pathways in medulloblastoma are the Sonic hedgehog (Shh) and the Wingless (Wnt) pathway. A recent genome-wide sequencing study in MB identified somatic mutations in both pathways, confirming their importance for MB development (244).

Abnormalities of the chromosome 17 are the most common chromosomal aberrations in MB. The most frequent chromosomal aberration found in medulloblastomas is the presence of isochrome i(17q), in 40%-60% of the cases accompanied by loss of 17p (245). The presence of i17q in tumours is associated with a poor clinical outcome, suggesting that it may contribute to the development of aggressive variants of MB (246).

Gene amplification in *MYC* or *MYCN* has been reported in 4-8% of MBs (247) and is associated with the more aggressive LC/A phenotype and poor outcomes (248). Interestingly, Shh and Wnt tumours rarely display abnormalities in chromosome 17 or amplification in *MYC* or *MYCN*.

Gain of chromosome 7 affects approximately 40% of cases (249). Monosomy of chromosome 6 frequently occurs in tumours with favourable prognosis, mainly classic subtype (250). Other chromosomal abnormalities that have been identified are loss of chromosome 10q, 7q and 16q.

MOLECULAR CLASSIFICATION OF MB SUBGROUPS

The currently used classification schemes of MB are mainly based on characteristic histopathological features defining several subtypes of MB, such as desmoplastic/nodular MB, MBEN (MB with extensive nodularity), classic MB, large-cell MB, and anaplastic MB (247, 251). However, due to the developments in the ability to monitor transcription across the genome, several groups have started to sub-classify MBs according to differences within their transcriptome (252). This transcriptional approach for tumour sub-classification underlies the assumption that tumours with similar transcriptomes will behave in biologically and clinically similar manner. This system is not applied in the clinics yet.

But recently, a new sub-classification for MB has been published according to a consensus conference, where members of many laboratories working in the field agreed on four principal molecular subgroups of medulloblastoma (253). These four sub-groups were named as follows: Wnt, Shh, 3, and 4, with the Wnt- and Shh-group named for the signalling pathway thought to play a prominent role in the pathology of the disease development in the particular subgroup. Since less is currently known about the biology of the remaining subgroups, the agreement was to apply unspecific names until the biological background underlying these subgroups was better defined (253). The four sub-groups show clearly distinct characteristics in terms of histology, demographics, DNA copy number

alterations, and clinical outcome (254) and were highly recommended to be employed in the clinics (253).

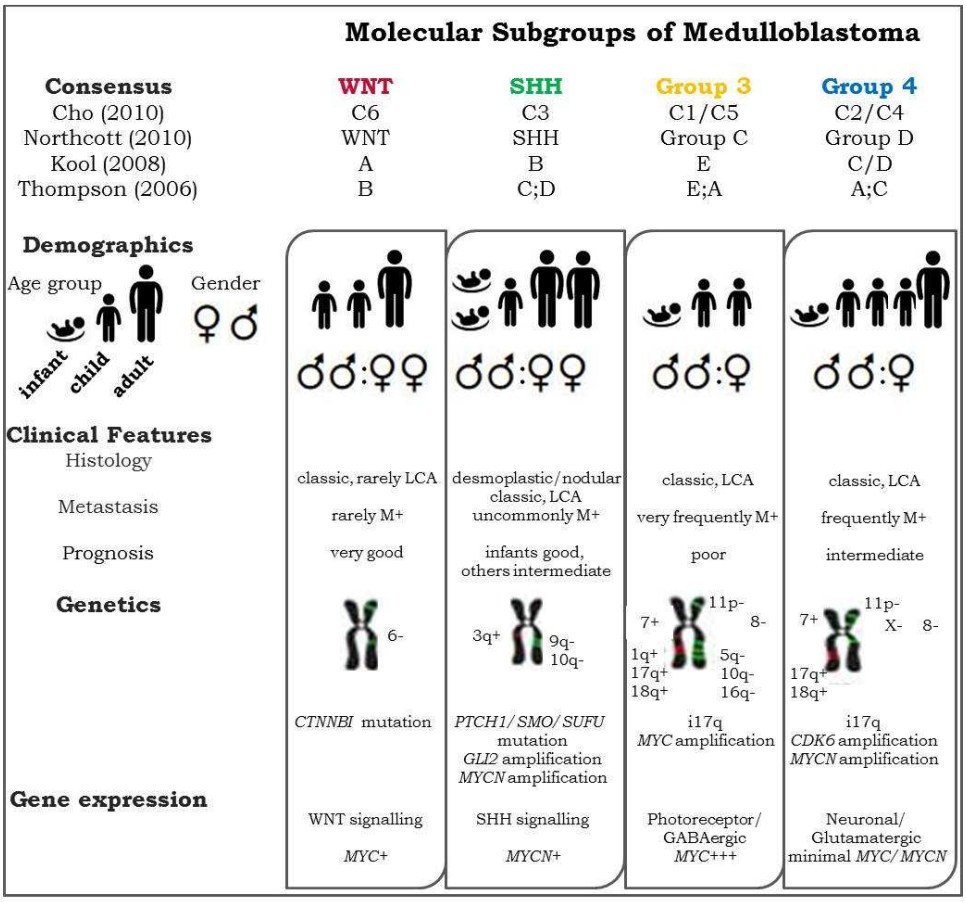

Figure 7 Genetic Alterations and Molecular Subgroups of Medulloblastoma. Content adapted from (253)

3.7 The Insulin-Like Growth Factor I Receptor

The insulin-related signalling system, consisting of three distinct receptors (insulin receptors (IRs), insulin-like growth factor receptors (IGF-IR and IGF-IIR (also known as IGF-II/mannose 6-phosphate receptor (M-6-PR)), two hybrid receptors, the signalling factors insulin, IGF-I, and IGF-II, as well as several circulating IGFBPs (insulin-like growth factor binding proteins 1-6) (255-257), has a critical role in growth, development, and metabolism. This phylogenetically conserved system evolved millions of years ago, predating the appearance of vertebrates. In human, the IR and IGFRs are widely expressed in many tissues and cells. Even though IGF-IR shows high homology to IR, each receptor has its particular role in the regulation of biological responses, either controlling cell growth, differentiation and apoptosis (IGF-IR) or physiological processes such as glucose metabolism (IR) (258-260).

The content of this thesis will emphasize on the signalling axis involving the IGF-IR and the role of its downstream mediator, PI3K isoform p110α, in the embryonal tumours NB and MB.

The receptor tyrosine kinase IGF-IR is a transmembrane tetrameric receptor with two extracellular α-subunits and two transmembrane β-subunits that are linked by disulfide bonds. The α-subunits bind to IGF, while the two β-subunits show intrinsic tyrosine kinase activity (261).

The IGF-IR has a high affinity for IGF-I and IGF-II, which are single-chain polypeptide protein hormones 60% similar to insulin (261). Insulin, the main ligand for the IR, has an IGF-IR-binding affinity which is less than 1% of that of IGF-I (262). Ligand-mediated activation of IGF-IR triggers the autophosphorylation of tyrosine residues within the kinase domain of the β-subunit, leading to the recruitment of the adaptor proteins IRS1 and Shc to the receptor β-subunit intracellular domains (263, 264). Phosphorylation of IRS1 activates the PI3K/Akt signalling pathway, the Shc adaptor triggers the activation of the RAS/RAF/MAPK pathways, thus controlling cell proliferation, differentiation and cell migration, but also regulating the apoptotic machinery (265).

The biological role of IGF-IR signalling in normal cells and mammalian development has been shown *in vitro* and *in vivo*, and reviewed numerous times in the past. Its role in normal growth control is convincingly demonstrated by the incidence of the congenital Laron-Syndrome also called Laron-type dwarfism, which is characterized by impaired development and growth and caused by low levels of IGF-I and its specific IGFBP. Additionally, mice harbouring a homozygous deletion of the IGF-IR gene are just half of the size compared to wild-type mice at the time of birth and die shortly after because of severe organ hypoplasia (266).

Deregulation of the IGF-IR and its downstream signalling mediators have been demonstrated to contribute to carcinogenesis. Epidemiological studies suggested the correlation of high levels of IGF-I and IGF-II with a higher risk of cancer (264), whereas patients harbouring a hereditary deficiency of IGF-I seemed to be protected from cancer development (267). High expression levels of the IGF-IR have been found to be present in several tumours and to be involved in tumour formation and metastasis. Therefore, the IGF-IR/IGF signalling system has become a promising target for the development of cancer therapeutics and numerous drug candidates are undergoing clinical trials.

3.8 The IGF-IR/PI3K Signalling Axis in Neuroblastoma & Medulloblastoma

Neuroblastoma. Insulin-like growth receptor signalling in neuroblastoma has been intensively studied in context of cell proliferation, survival, and motility (268-270).

Functional IGF-IR is expressed on the surface of NB cells and was associated with tumourigenesis and metastasis (271). Even though mutations in the *IGF1R* gene have not been reported, the gene encoding its corresponding ligand IGF-II was shown to be abnormally expressed in NB tumour samples. IGF-I and IGF-II stimulate the proliferation in NB cells and IGF-II particularly acts in an autocrine/paracrine manner on the IGF-IR and triggers NB cell differentiation, cytoskeletal rearrangement, angiogenesis, and suppresses apoptosis via the PI3K pathway, contributing to tumourigenesis (272).

Even though activating *PIK3CA* mutations are common events in many cancers, they do not frequently occur in neuroblastoma. One report described 2 point mutations in 42 NB samples and 27 NB cell lines (273, 274). Even though around 80% of the *PIK3CA* mutations map to one of three hot spots, which are characterized by the gain of a single amino acid (E542K, E545K and H1047R), none of the aberrations in NB was found to lie within these particular regions, but were located within the helical or kinase domain. Subsequently, another study could identify a rarely seen somatic mutation located in exon 5 and reported PI3K p110α expression in 92% of NB tissue sections (273). In contrast, a recently published report could not confirm any of these results and demonstrated the absence of any *PIK3CA* mutations in the panel of NB samples tested. Amplifications in the *PIK3CA* locus (3q26) could also not be identified by screening a panel of 42 NB cell lines (275). As in the case of *PIK3CA*, genetic alterations in *PIK3CD* have been shown to be rare (276), only 2/46 primary NB samples harboured genetic alterations in the gene encoding p110δ. Elevated mRNA levels of *PIK3CD* were shown to occur in patients younger than 1 year of age and interestingly, the results of the same study revealed a negative correlation with *MYCN* amplification (277). Genetic alterations of *PTEN* have been rarely reported in neuroblastoma and association with the incidence of an aggressive tumour phenotype could not be noticed (273, 278, 279). Downstream of the PI3K phosphorylation of the serine/threonine kinase Akt was shown to be a marker for poor prognosis in NB, whereas the activation of S6 or Erk did not display prognostic values (280).

Medulloblastoma. Activation of the IGF-IR system has been reported to be present in human and mouse MB cell lines, as well as in MB biopsies, and for instance IGF-II is known control the proliferation of normal cerebellar neural precursors, as well as MB cells via IGF-IR. The activated form of IGF-IR could be detected in approximately 60% of MB patient samples, but did not show any correlation with patient prognosis (281). Interestingly, an inverse correlation of phosphorylated IGF-IR presence and Trk-C protein expression, which was already proposed to be a molecular marker for favourable prognosis in MB, was reported (282). Autocrine IGF-IR/IGF-I signalling promotes proliferation signals via the MAPK pathway in MB cells. Activation of the PI3K/Akt signalling cascade was detected in primary tumours and MB cell lines. Whereas no amplifications of the *PIK3CA* gene could be detected in one report and another study demonstrated *PIK3CA* mutations only at low frequency (5%) (283, 284), overexpression of the *PIK3CA* gene and of its product PI3K p110α was observed in a subset of MB tumour samples and cell lines, often accompanied by constitutive activation of Akt (284). The importance of p110α was proven, as its inhibition led to a decrease in cell proliferation and augmented the effects of chemotherapy in a cell line model (284). The remaining class I_A PI3K (catalytic) isoforms p110β and p110δ did not display significant increases in of the expression levels (284), whereas the class I_B isoform PI3K p110γ showed overexpression in subset of medulloblastoma samples and cell lines and its inhibition led to sensitization to chemotherapeutic treatment *in vitro* (285). PTEN expression was found to be reduced in medulloblastoma (286, 287). PTEN mRNA and protein expression levels were significantly lower in primary medulloblastoma

samples, when compared to normal cerebellar tissue. In 50% of the tumour samples, reduction of PTEN expression was found to be associated with PTEN promoter hypermethylation (286). Homozygous loss of PTEN at chromosome 10q was demonstrated in 32% of medulloblastomas (287). However, a different study found that the PTEN protein was expressed at higher levels in medulloblastoma samples and cell lines as compared to control samples (284).

Targeting PI3K signalling with pharmacological inhibitors has become an important experimental therapeutic approach for cancer. Targeting the RTK/PI3K/Akt/mTOR cell survival axis and its downstream mediators with small molecule inhibitors (tyrosine kinase inhibitors, TKIs) is, beside treating cancer with classical chemotherapeutical agents, one of the most prominent approaches used in cancer therapy and has been reviewed in the past for various human cancers. One of the most prominent PI3K-inhibitors is the dual PI3K p110α/mTOR inhibitor PI103, which inhibited neuroblastoma growth *in vitro* and *in vivo* (288, 289). Apoptosis-resistance in NB cell lines, evoked by an IGF-I-induced activation of the PI3K/Akt signalling, could be completely reversed by the PI3K inhibitor LY294002 (280). In medulloblastoma cell lines, inhibition of the PI3K isoform p110α with the isoform specific inhibitors PIK75 and YM024 was reported to have anti-proliferative effects (284) and furthermore, molecules targeting the PI3K/Akt signalling pathway, such as a small-molecule inhibitor of the PDK1, affected β-catenin signalling via GSK3β inhibition, resulting in apoptosis and suppressed MB tumour growth (290).

4 RESULTS

4.1 SUBJECT OF INVESTIGATION

TARGETING THE PHOSPHOINOSITIDE 3-KINASE P110 ALPHA ISOFORM IMPAIRS CELL PROLIFERATION, SURVIVAL, AND TUMOUR GROWTH IN SMALL CELL LUNG CANCER

In this study we have investigated the role of the class I_A PI3K isoform p110α and the potential of targeting PI3K signalling in small cell lung cancer, which is the most aggressive of all lung cancer types and almost entirely related to smoking.

The PI3K/Akt/mTOR pathway has been demonstrated to play a key role in SCLC cell proliferation, survival, chemoresistance and migration. Mutations in *PIK3CA* and gene amplification were reported in primary SCLC, as well as increased expression of *PIK3CA* at the mRNA and protein level.

Thus, we hypothesized involvement of the class IA PI3K isoform p110α in SCLC biology being an important mediator for pro-survival signals in SCLC cell responses.

An immunohistochemistry analysis in primary SCLC tissue samples had revealed over-expression of the PI3K downstream target mTOR, leading to the question of the expression level of class I_A PI3Ks in SCLC patient samples, which was then also studied by immunohistochemical analysis.

Broad specificity PI3K/mTOR inhibitors have shown anti-tumour activity in SCLC models *in vitro* and *in vivo* (118). In view to these results, we sought to investigate the potential of isoform-specific inhibitors and RNAi targeting class I_A PI3K isoforms on SCLC cell responses. By targeting the class I_A PI3K isoforms with specific pharmacological inhibitors, such as PIK75 and YM024 (p110α), PI103 (p110α/mTOR), TGX221 (p110β), IC87114 (p110δ) or silencing their expression via siRNA and shRNA constructs we have investigated SCLC cell responses, such as PI3K downstream signalling activation, cell viability, cell cycle progression and survival, as well as mechanistic processes important for tumour growth and maintenance, such as angiogenesis.

In order to investigate whether the class I_A PI3K isoform p110α controls the expression of specific gene subsets in SCLC, we performed DNA microarray analysis in H69 cells treated with either vehicle, PIK75 targeting p110α, or TGX221 targeting p110β. Inhibition of p110α with PIK75 strongly affected gene expression in widespread functional categories, compared to p110β inhibition, which did not have any significant effect on the SCLC gene expression.

This results support a model, in which p110α is the most important class I_A PI3K in SCLC and raised the question of its downstream targets controlling SCLC cell responses upon p110α inhibition.

To gain insight into the transcriptional networks affected by silencing p110α in SCLC cells, we performed a biostatistics analysis of the gene expression data, using GeneGo. The transcriptional networks that were most significantly altered comprised HNF4α, SP1 and c-Myc, the estrogen receptor (ER) and also NFκB. We hypothesized NFκB to be a downstream target of p110α controlling the expression of Bcl2 and $BclX_{L,}$ found to be down-regulated by p110α inhibitors.

Furthermore, to confirm the *in vitro* results obtained in an *in vivo* model we performed a tumour growth assay on the CAM of chick embryos. SCLC cells were applied on the CAM in presence or absence of the p110α inhibitor PIK75 to investigate tumour formation, growth, and vascularization.
Together, our data strongly suggest the PI3K isoform p110α as a critical mediator for SCLC growth and cell survival processes.

NOVEL AGENTS TARGETING THE IGF-IR/PI3K PATHWAY IMPAIR CELL PROLIFERATION AND SURVIVAL IN SUBSETS OF MEDULLOBLASTOMA AND NEUROBLASTOMA

Embryonal tumours (ET) represent an important group of infant malignancies arising from different tissues of fetal origin. Among ET, the tumours of the central nervous system (CNS) account for approximately 20% of all childhood cancers, ranking second only to leukemias. They are highly malignant and characterized by poorly differentiated neuroepithelial cells with the potential capacity for divergent differentiation. The IGF-IR/PI3K pathway, fundamental for cell proliferation and survival, is known to be frequently altered and activated in neoplasia, including embryonal tumours.
Here, we have investigated the potential of targeting the axis of the IGF-IR and PI3K signalling in two common cancers of childhood: 1) Neuroblastoma (NB), the most common extracranial tumour in children causing around 15% of all childhood cancer-related mortality and 2) Medulloblastoma (MB), the most frequent childhood brain tumour. Activation and deregulation of the IGF-IR/PI3K signalling system has been reported to be present in NB and MB, including autocrine signalling loops, over-expression and genetic alteration found in genes encoding PI3K signalling components.
By treating NB and MB cells with R1507 (Roche), a specific humanised monoclonal antibody against the IGF-IR or targeting the class I_A PI3K p110α with the specific inhibitor PIK75, we wanted to evaluate their impact on cell proliferation, survival, expression and phosphorylation state of IGF-IR/PI3K downstream signalling targets, as well as their effects on chemoresistance.

4.2 MANUSCRIPTS

TARGETING THE PHOSPHOINOSITIDE 3-KINASE P110A ISOFORM IMPAIRS CELL PROLIFERATION, SURVIVAL AND TUMOUR GROWTH IN SMALL CELL LUNG CANCER

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Grant Support: Association for International Cancer Research, Wilhelm Sander-Stiftung, Novartis Stiftung für Medizinisch-Biologische Forschung, Department of Clinical Research of the University of Bern.

Running title: Targeting PI3K signalling in SCLC
Keywords: apoptosis; Bcl-2; PI3K; proliferation; small cell lung cancer

Conflicts of interest: The authors declare that there is no conflict of interest.

This manuscript has been resubmitted after revsions to the AACR journal *Clinical Cancer Research*.

Abstract

Purpose: The phosphoinositide 3-kinase (PI3K) pathway is fundamental for cell proliferation and survival and is frequently altered and activated in neoplasia, including carcinomas of the lung. In this study we investigated the potential of targeting the catalytic class I_A PI3K isoforms in small cell lung cancer (SCLC), which is the most aggressive of all lung cancer types. Furthermore, SCLC is known to commonly display Bcl-2 over-expression or *TP53* mutations which could lead to defects in the apoptotic machinery. Here, we hypothesized the PI3K p110α to have a regulating role in the expression of proteins of the Bcl-2 family.

Experimental Design: The expression of PI3K isoforms in patient specimens was analyzed. The effects on SCLC cell survival and downstream signaling were determined following PI3K isoform inhibition by selective inhibitors or down-regulation by small interfering RNA.

Results: Over-expression of the PI3K isoforms p110α and p110β and the anti-apoptotic protein Bcl-2 was shown by immunohistochemistry in primary SCLC tissue samples. Targeting the PI3K p110α with RNA interference (RNAi) or selective pharmacological inhibitors resulted in strongly affected cell proliferation of SCLC cells *in vitro* and *in vivo*, while targeting p110β was less effective. Inhibition of p110α also resulted in increased apoptosis and autophagy, which was accompanied by decreased phosphorylation of Akt and components of the mammalian target of rapamycin (mTOR) pathway, such as the ribosomal S6 protein, and the eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1). A DNA microarray analysis revealed that p110α inhibition profoundly affected the balance of pro- and anti-apoptotic Bcl-2 family proteins. Finally, p110α inhibition led to impaired SCLC tumor formation and vascularization *in vivo*.

Conclusion: Together our data demonstrate the key involvement of the PI3K isoform p110α in the regulation of anti- and pro-apoptotic proteins of the Bcl-2 family and its particular role in the regulation of multiple tumor-promoting processes in SCLC.

Translational relevance

Small cell lung cancer (SCLC) is a common form of lung cancer and is associated with very unsatisfactory survival rates. Novel therapies are thus urgently required and will arise from a better understanding of the disease biology. We were interested in gaining further insight into the potential of targeting PI3K isoforms in SCLC. In this report, we show that the catalytic p110α isoform is overexpressed in a subset of primary SCLC samples. The growth of SCLC cells was impaired on targeting p110α using RNA interference or specific pharmacologic inhibitors. Inhibition of p110α also induced apoptosis and autophagy, which was paralleled by a decrease in the expression levels of anti-apoptotic Bcl-2 family proteins. Importantly, SCLC tumors treated with p110α inhibitors displayed reduced proliferation and enhanced apoptosis *in vivo*. Together, the results presented in this study show that specific p110α inhibitors may in the future represent new drugs for SCLC.

Introduction

Lung cancer is a major cause of death in the developed world and the commonest cancer killer in men. Small cell lung cancer (SCLC) represents about 13-15% of all cases of lung cancer and is strongly associated with cigarette smoking. Combinatorial chemotherapy regimens with etoposide and platinum-based agents, as well as radiotherapy are commonly used for the treatment of SCLC patients. However, an initial therapeutic responsiveness is usually followed by disease recurrence within less than 1 year and therefore the overall 5-year survival rate is <5%. Consequently, novel therapeutic strategies are urgently required for SCLC. In the past years, an increasing number of molecular alterations involved in SCLC pathogenesis have been reported, including ectopic expression of neuroendocrine regulatory peptides, up-regulation of anti-apoptotic Bcl-2 proteins, overexpression of *myc* family oncogenes and extracellular matrix proteins, as well as genetic abnormalities in the tumor suppressor genes *TP53* and *RB* (108, 156). In addition, it has been shown that polypeptide growth factors such as hepatocyte growth factor (HGF), fibroblast growth factor-2 (FGF-2), insulin-like growth factor-1 (IGF-1) and stem cell factor (SCF) control key biological responses in human SCLC cells, including growth and proliferation, chemoresistance, and migration (106, 147, 149, 182). Downstream of activated polypeptide growth factor receptors, activation of two major intracellular signaling cascades, the phosphoinositide-3 kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) and the mitogen-activated Erk kinase (MEK)/extracellular signal-regulated kinase (Erk) pathway, have been found to be involved in the survival and proliferation of SCLC (147, 149, 182, 187, 291). Furthermore, it has been reported that mTOR and the ribosomal protein S6 kinases (S6K) are over-expressed in SCLC cells compared with normal human type II pneumocytes (147). Therefore, targeting these pathways with novel selective pharmacological inhibitors may lead to the development of more effective therapies for SCLC.

The PI3K signaling pathway controls key cellular responses, such as cell growth and proliferation, survival, migration and metabolism. Over the last decades, it has been recognized that this intracellular signaling pathway is frequently activated by genetic and epigenetic alterations in human cancer, including lung cancer. The PI3K family of signaling enzymes comprises eight catalytic isoforms, which are subdivided into three classes. The class I_A PI3K isoform p110α is considered to be a validated drug target in human cancer (5, 11, 292), in particular because activating mutations in the *PIK3CA* frequently occur in human cancer. In SCLC, amplification and mutations of the *PIK3CA* gene were identified and the p110α and p110β isoforms are over-expressed in cell lines, additionally joined by deregulation of the PI3K/Akt/mTOR pathway (293).

Here we report for the first time that targeting the class I_A PI3K isoform p110α blocks SCLC cell growth and survival *in vitro* and *in vivo* and present evidence that this isoform plays a crucial role in Akt/mTOR pathway activation and Bcl-2 family proteins expression.

Materials and methods

Antibodies and reagents

Antibodies: Caspase 3, poly-(ADP-ribose)-polymerase (PARP), Akt, PI3K p110β (Santa Cruz Biotechnology), p-Akt^{Ser473}, p-Bcl2^{Ser70}, Bcl2, BclX_L, Bax, Bad, p-4EBP1$^{Thr37/46}$, p-S6$^{Ser235/236}$ or p-S6$^{Ser240/244}$, S6 protein, PI3K p110α (Cell Signaling Technology), β-actin (Sigma Aldrich), Mcl-1, NFκB (Epitomics).

Etoposide, chloroquine, z-VAD-FMK, IKK inhibitor (wedelolactone)(Calbiochem). RAD001 was supplied by the Novartis Institutes for BioMedical Research Basel, Oncology, Switzerland. The PI3K inhibitors PIK75, YM024, TGX221, PI103 were the kind gift of Prof. Shaun Jackson.

Cell lines, cell culture, and cell proliferation

SCLC cell lines were obtained from the American Type Culture Collection (Suppl. Table 3). The human SCLC cell lines H69, H209, H510, and SW2 were cultured in RPMI medium containing 10% heat-inactivated FCS. Cell viability was analyzed by MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Promega).

Reverse transcription-PCR and TaqMan analysis

Total cellular RNA was extracted using the RNeasy Mini Kit (QIAGEN) according to the manufacturer's instruction. For each reverse transcription-PCR, 1 µg of total RNA was used with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The reaction conditions were used according to the manufacturer's protocol. TaqMan Gene Expression Assays for the PI3Ks p110α and p110β, as well as for VEGFA were done according to the manufacturer's instructions (Applied Biosystems). As internal control the expression of GAPDH was analyzed in each sample, separately.

Immunohistochemistry

Immunoreactivity was evaluated on commercial TMA sections of SCLC (Biomax LC10010 (2 cores; Female 9 (22.5%), Female age 32-66 yo (mean value 52.5); Male 31 (77.5%), Male age 34-76 yo (mean value 53.0); Stage I 11, Stage II 20, Stage IIIa 7, Stage IIIb 2; Node negative 12, Node positive 28 (22 N1 and 6 N2)) using the PI3K p110α (Cell Signaling Technology, 4249), p110β (Abcam, ab55593) and Bcl2 (Cell Signaling) antibodies in a modification of the antigen retrieval technique (294). The antibodies were used at 1:200 dilution for 1 hour at room temperature and then processed with Polymer-HRP Kit (BioGenex, San Ramon CA) with diaminobenzidine development and Mayer haematoxylin counterstaining. Breast cancer tissue was used as positive external control. Negative controls were obtained by omitting the primary antibody. Scoring A semi quantitative immunohistochemical score (IHS) was used including assessment of both the percentage of positive

cells and the intensity of staining. For the intensity, a score of 0 to 3, corresponding to negative, weak, moderate and strong positivity, was recorded. The range of possible scores was thus 0 to 300. IHS and similar semi quantitative scoring systems have been successfully used for TMA evaluation. Each core was scored individually. One observer scored all cases, which were rechecked randomly by the same investigator after a period of time.

Serial sections of paraffin-embedded CAM tumors were dewaxed in xylol and rehydrated through graded dilutions of ethanol. Antigen recovery was performed with citrate buffer (10 mM) in a microwave (800 watts) for 7 min. Endogenous peroxidase activity was blocked with 3% H_2O_2. Sections were incubated in a humid chamber at 4 °C overnight with or without (negative controls) active Caspase 3 (Abcam, ab2302; recognizes the cleaved active p17 fragment) and Ki67 (Novus Biologicals, NB110-57147) antibodies. Biotin-conjugated goat anti-Rabbit (Dako, E0432) and avidin-biotin-horseradish peroxidase complex (Vector Laboratories, Vectastain ABC Kit, PK6100) were used. Bound peroxidase was detected with 3, 3'-diaminobenzidine substrate. Sections were subsequently counterstained with hematoxylin and mounted in Aquatex (Merck).

Transient transfection and stable transduction of SCLC cells

SCLC cells were transiently transfected with either non-targeting small interfering RNA (siRNA) or SMARTpool siRNA duplexes targeting the PI3K p110α or PI3K p110β, using the Amaxa Nucleofector system (Amaxa Biosystems), according to the manufacturer's protocol. Briefly, 1.5 x 10^6 SCLC cells grown in RPMI/10% FCS were transfected with 6 µL of 20 µmol/L siRNA in 100 µL of Nucleofector Solution V using the program T-16 on the Amaxa Nucleofector. Following transfection, cells were transferred into RPMI/10 % FCS overnight before they were used for experiments.

Lentiviral vectors expressing small hairpin (sh)RNA constructs specificially targeting the PI3K p110α (Sigma-Aldrich) were tested for stable knockdown efficiency. The constructs shPI3KC_2892 and shPI3KC_3433 were chosen for further experiments since they most efficiently silenced p110α. The non-targeting vector SHC002 was used as control. Lentivirus production and transduction of cells was done as described before (295). Vectors contained a puromycin-resistance gene and stably transduced cell populations were selected for two weeks using puromycin at the concentration 1.5 µg/ml.

In vivo *experiments*

The effect of the PI3K inhibitor PIK75 on the growth and SCLC tumor formation *in vivo* were assessed on the chorioallantoic membrane (CAM) of chick embryos (296)**.** Briefly, 3 x 10^6 H69 SCLC cells were placed in RPMI containing 50% BD Matrigel Matrix (BD Biosciences) on the CAM on embryonic day 9. Increasing concentrations of the p110α inhibitors PIK75 were deposited with the SCLC cells. Controls received the solvent of the corresponding drug. CAMs were examined for vessel formation under a stereomicroscope. Tumor size and density of vessels per area around the tumor were

determined using the software Vessel_tracer (297). The application of Matrigel without/with PIK75 on the CAM was used as negative control.

SDS-PAGE and Western blotting

The assays were done as described before (182).

DNA microarray

The cDNA microarray analysis was performed in collaboration with the Functional Genomic Center of the University of Zurich. Gene expression data were obtained by hybridizing Human Genome U133 Plus 2.0 Affymetrix GeneChips arrays, on which >54000 transcripts were represented. Each experiment represented a group of three independent biological replicates. Raw data generated by the GCOS Software (Affymetrix) were processed by using the RMA method (298) and further statistically analyzed by using the software R and applying Student's t-test. The GeneGO MetaCore (GeneGO, St Joseph, MI, USA) was used to define functional annotations for the selected genes, thus assigning them to ontological categories for association with relevant biological processes and pathways.

Results

Expression of the PI3K p110α and p110β and the anti-apoptotic protein Bcl2 in SCLC patient samples

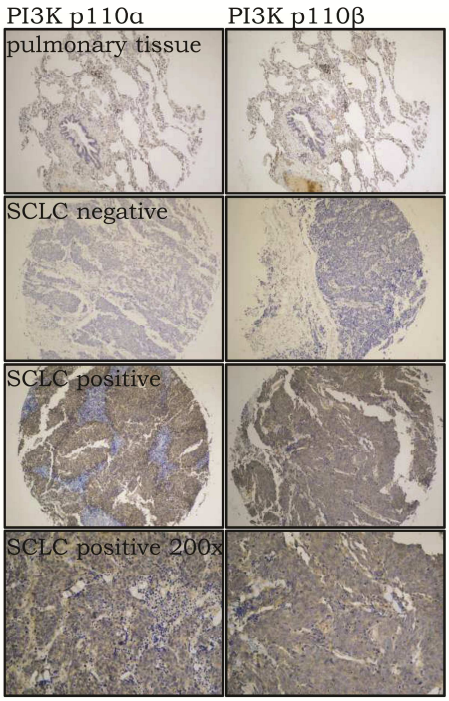
Our previous work in SCLC cell lines had revealed that class I_A PI3K isoforms are over-expressed in comparison to type II pneumocytes, which are one of the precursors of SCLC. To investigate whether this finding could be confirmed in primary tumors, a SCLC tissue microarray was stained with antibodies specific for p110α or p110β (Fig. 8A). This analysis revealed that, while normal lung tissue did not express detectable levels of either PI3K isoforms, p110α and p110β displayed enhanced expression in subsets of primary SCLC (Fig. 8A-B). Around 25% of SCLC tumors had over-expression of p110α, while 18% of cases were positive for p110β (Fig. 8B). In general, SCLC tumors did not display concomitant over-expression of p110α and p110β and was only present in 5% of the tumors. Additionally, the expression of the anti-apoptotic protein Bcl2 was assessed in SCLC patient samples. The IHC staining of a TMA with a specific antibody revealed a high Bcl2 over-expression in ~90% (35/39 cases) of the SCLC patient samples (Fig. 8C+D) compared to normal lung tissue, which did not display detectable levels of Bcl2.

Class I_A PI3K p110α inhibition or silencing blocks cell proliferation and Akt/mTOR signaling

Previous work had shown that targeting the PI3K/Akt/mTOR pathway by using small molecule inhibitors impaired SCLC proliferation *in vitro* and *in vivo*. Therefore, we investigated the impact of isoform-specific inhibitors of class I_A PI3K isoforms on the proliferation of SCLC cell lines. The potent p110α inhibitor PIK75 (IC_{50} in vitro = 7.8±1.7 nM) (299) significantly impaired the proliferation of 4 SCLC cell lines with IC_{50} values in the range of 50-100 nM (Fig. 9A). The p110α inhibitor YM024, which is much less potent against the enzyme *in vitro* (IC_{50} in vitro = 0.3 μM), also impaired the proliferation of the SCLC cell line panel, but at higher concentrations (Fig. 9A). The potent p110β inhibitor TGX-221 (IC_{50} in vitro = 8.5±0.9 nM) (299) only partially impaired the proliferation of SCLC cell lines, but at high concentrations (Fig. 9B). In contrast, the p110δ inhibitor IC87114 did not significantly impair the proliferation of the cell lines under study (Fig. 9B). We also evaluated the impact of the dual p110α/mTOR inhibitor PI103 in the panel of SCLC cell lines. PI103 displayed anti-proliferative activity against the SCLC panel in vitro, with IC50 values in the range of 100-500 nM (Suppl. Fig. 1A).

The impact of the selective PI3K inhibitors on the activation status of the Akt/mTOR pathway in SCLC cell lines was then investigated by Western blot analysis (Fig. 9C). PIK75 and PI103 strongly affected the activation status of Akt and the phosphorylation of the mTOR downstream targets ribosomal S6 protein and 4E-BP1 (Fig. 9C; Suppl. Fig. 1B). YM024 also impaired Akt activation and partially inhibited mTOR pathway activation (Fig. 9C; Suppl. Fig. 1B). In contrast, TGX221 was less effective at blocking the activation of the Akt/mTOR pathway (Fig. 9C; Suppl. Fig. 1B).

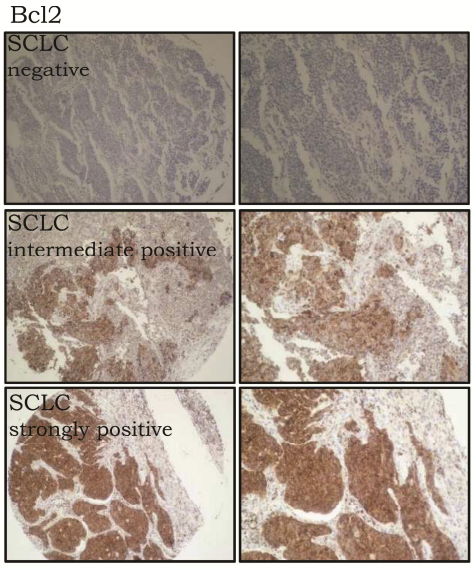
A



B

	number of specimens	positive	%
p110α			
normal lung tissue	*20*	*0*	*0*
SCLC patient samples	*80*	*20*	*25*
T1	*26*	*3*	*12*
T2	*46*	*15*	*33*
T3	*4*	*1*	*25*
T4	*4*	*3*	*75*
p110β			
normal lung tissue	*20*	*0*	*0*
SCLC patient samples	*80*	*14*	*18*
T1	*26*	*4*	*15*
T2	*46*	*10*	*22*
T3	*4*	*1*	*25*
T4	*4*	*0*	*0*

C



D

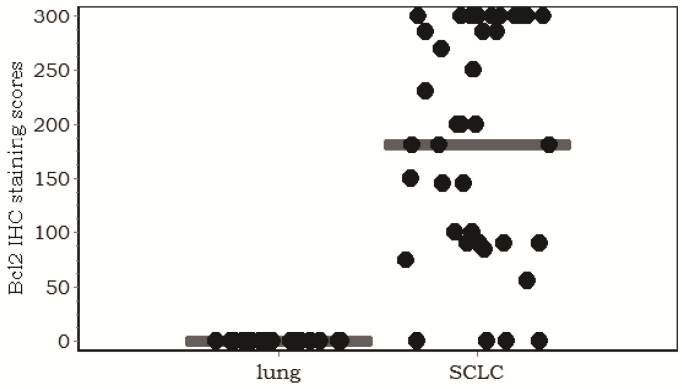


Figure 8 Expression of the PI3K p110α and p110β an the anti-apoptotic protein Bcl-2 in SCLC patient samples. (a) Immunostaining with PI3K p110α and p110β showing negative SCLC staining and representative p110α and p110β SCLC positive staining, compared to normal lung tissue. (b) Analysis of the IHC staining PI3K p110α and p110β in SCLC patient samples.(c)Immunostaining with Bcl-2 showing negative and representative Bcl-2 intermediate and strongly positive SCLC staining. (b) The mean immunohistochemical staining scores (IHC scores) Bcl-2 in 20 paraffin-embedded specimens each of normal lung tissue and of 40 SCLC paraffin-embedded specimens. IHC scores = percentage of positive cells x staining intensity (for details see “Material and Methods”).

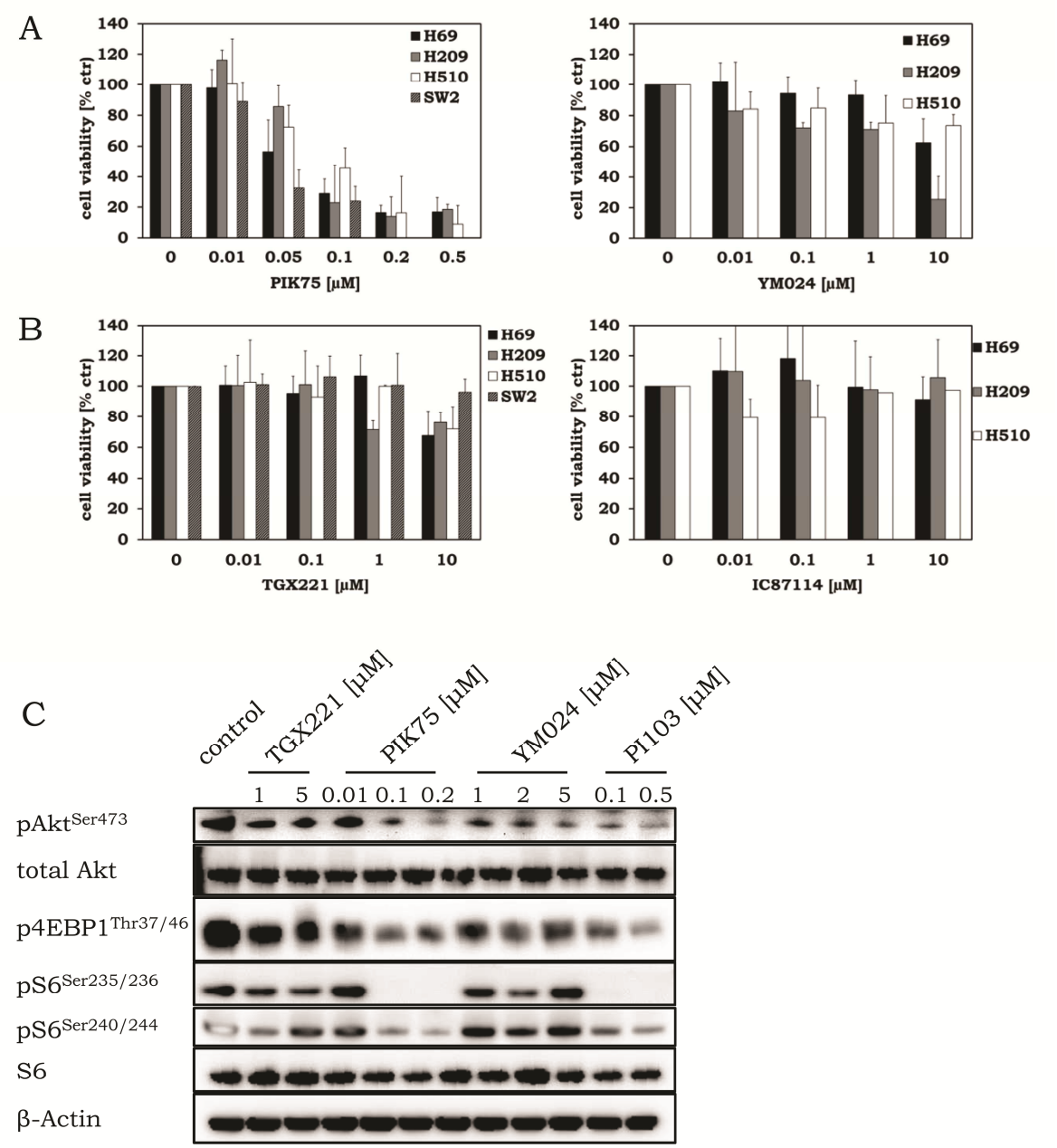


Figure 9 PI3K inhibition blocks cell viability and PI3K downstream signaling. (a+b) The SCLC cell lines H69, H209, H510, and SW2 were incubated with increasing concentrations of the class I PI3K inhibitors PIK75, YM024 (a), TGX221, and IC87114 (b) in serum-containing medium. Cell viability was assessed using the MTS assay after 3 days. The data are mean with SD from four replicates and at least three independent experiments. (c) H69 cells were incubated with increasing concentrations of the PI3K p110α inhibitors PIK75, YM024, and PI103 (PI3K p110α/mTOR inhibitor) and the PI3K p110β inhibitor TGX221. After 24 hours the cells were harvested and whole cell lysates analyzed by SDS-PAGE and Western blotting for the proteins indicated.

To validate our findings with pharmacological inhibitors, we used RNA interference to down-regulate the expression of p110α or p110β in SCLC cell lines. Transient down-regulation of p110α strongly impaired the activation of Akt and phosphorylation of the ribosomal S6 protein, while p110β silencing was ineffective (Fig. 10A). In addition, p110α silencing induced a significant decrease in the proliferation of SCLC cells (40% reduction), while p110β down-regulation was less effective (25% reduction) (Fig. 10B). We also used lentiviral delivery of short hairpin RNAs targeting p110α in

SCLC cell lines. Also this approach resulted in stable silencing of p110α, robust affected PI3K downstream signaling (Fig. 10C) and SCLC cell proliferation was partially impaired by shRNA targeting of p110α (Fig. 10D).

Together these results show that targeting p110α selectively impairs cell proliferation and activation of the Akt/mTOR pathway in SCLC cell lines *in vitro*.

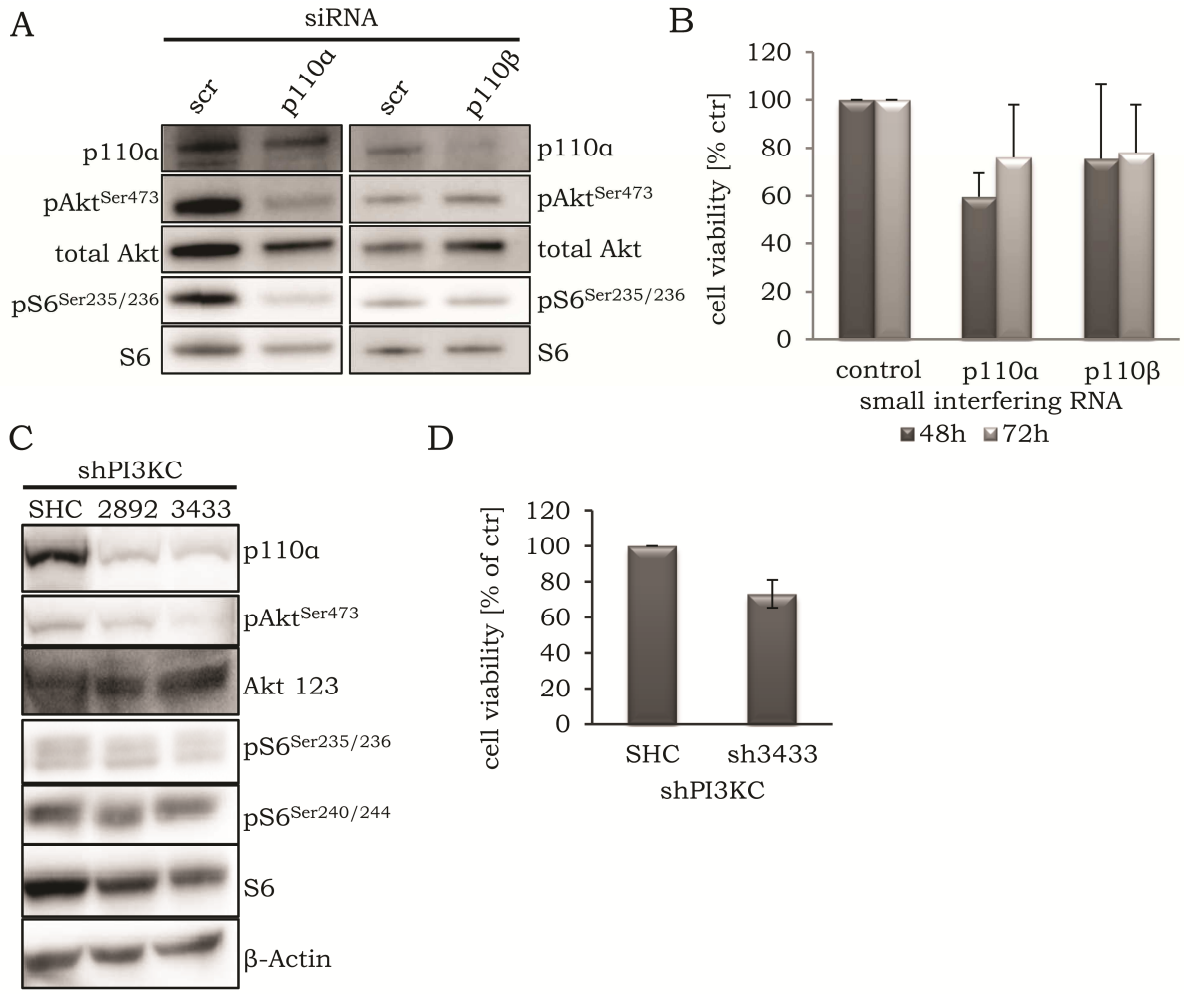


Figure 10 Silencing of p110α but not p110β affects cell viability and PI3K downstream signaling activation. (a+c) H69 cells were transiently transfected with siRNA constructs targeting p110α or p110β, or non-targeting scrambled control (a) or p110α was stably silenced by the lentiviral delivery of shRNA constructs (c). Cell lysates were analyzed by SDS-PAGE and Western blotting with antibodies for the proteins indicated. (b+d) Cell viability of H69 cells transiently (b) or stably (d) transfected with constructs targeting p110α or p110β was assessed using the MTS assay after 2 and 3 days. A non-targeting construct was used as control.

Class I_A PI3K p110α inhibition induces apoptosis and autophagy in SCLC cell lines

Previous work had shown that targeting the PI3K/Akt/mTOR pathway by using small molecule inhibitors (187, 300) or RNAi induced apoptosis in SCLC cell lines. Therefore, we investigated the impact of isoform-specific inhibitors of class I_A PI3K isoforms on the survival of SCLC cell lines. Treatment of a panel of SCLC cell lines with PIK75 or YM024 induced cleavage of PARP and reduction of pro-caspase 3, which are markers of apoptosis induction (Fig. 11A), whereas TGX221 was less efficient at inducing apoptosis (Fig. 11A). Apoptosis induction upon p110α inhibition seemed

to be stronger in the SCLC cell lines H69 and H209, compared with the effects observed in H510 (intermediate) and SW2 (low) (Fig. 11A). The caspase inhibitor zVAD-FMK was able to rescue 20% of H69 cells from PIK75-induced apoptosis and 10% of the TGX221-induced apoptosis ($p<0.01$) (Fig. 11B). Because the strongly affected cell viability in SCLC cells due to p110α inhibition could not only be explained by apoptotic cell responses we further wanted to investigate other mechanisms, such as autophagy, responsible for SCLC cell death. Whereas the inhibition of p110α with PIK75 or YM024 was able to induce increased conversion of LC3-I to LC3-II, which is an indicative for autophagic activity, it could be less observed in p110β-inhibited cells (Fig. 11C, Suppl. Fig. 2). ATG5, another protein used as typical marker for autophagy, was also expressed in SCLC cells. Compared to the vehicle-treated control no or just a slightly enhanced expression could be observed in PIK75-treated H69 cells (Fig. 11C). Interestingly, at later time points, a decrease in ATG5 expression could be observed (data not shown). Chloroquine, a drug actually used as anti-malaria treatment, was discovered to inhibit autophagic activity due to deacidification of lysosomes followed by accumulation of ineffective autophagic vesicles. Also in H69 cells treated with the PI3K inhibitors PIK75 and TGX221 chloroquine was able to rescue 20% (and 10%) of the autophagy-induced decrease in cell viability (Fig. 11D), suggesting additive roles or crosstalk between autophagy and apoptosis in p110α-induced cell death.

***Inhibition of the PI3K p110α impairs SCLC tumor formation and vascularization* in vivo**

The overview of data collected from our present experiments and the known literature indicates an important role of p110α in SCLC cell growth and survival processes *in vitro* and *in vivo*. To further investigate its impact on tumor formation and maintenance, an *in vivo* assay was employed to grow vehicle- and PIK75-treated H69 cells on the chorioallantoic membrane (CAM) of chick embryos. Tumor formation was clearly impaired upon PIK75 treatment pointed out by strongly reduced tumor size and tumor weight compared to the control tumors (Fig. 12A). Additionally, IHC of PIK75-treated tumor sections showed a decreasing expression of the proliferation marker Ki67 and a rising expression of the apoptotic marker cleaved caspase 3 with increasing PIK75 concentrations (Fig. 12C). Finally, p110α inhibition was able to partially prevent the vascularization on the CAM around the tumorigenic area as measured by the vessel density (Fig. 12B). Another hint leading to the assumption of an impaired tumor vascularization through p110α inhibition could be the reduced VEGFA expression found in DNA-microarray- and quantitative RT-PCR analysis of PIK75-treated H69 cells (Suppl. Fig. 3).

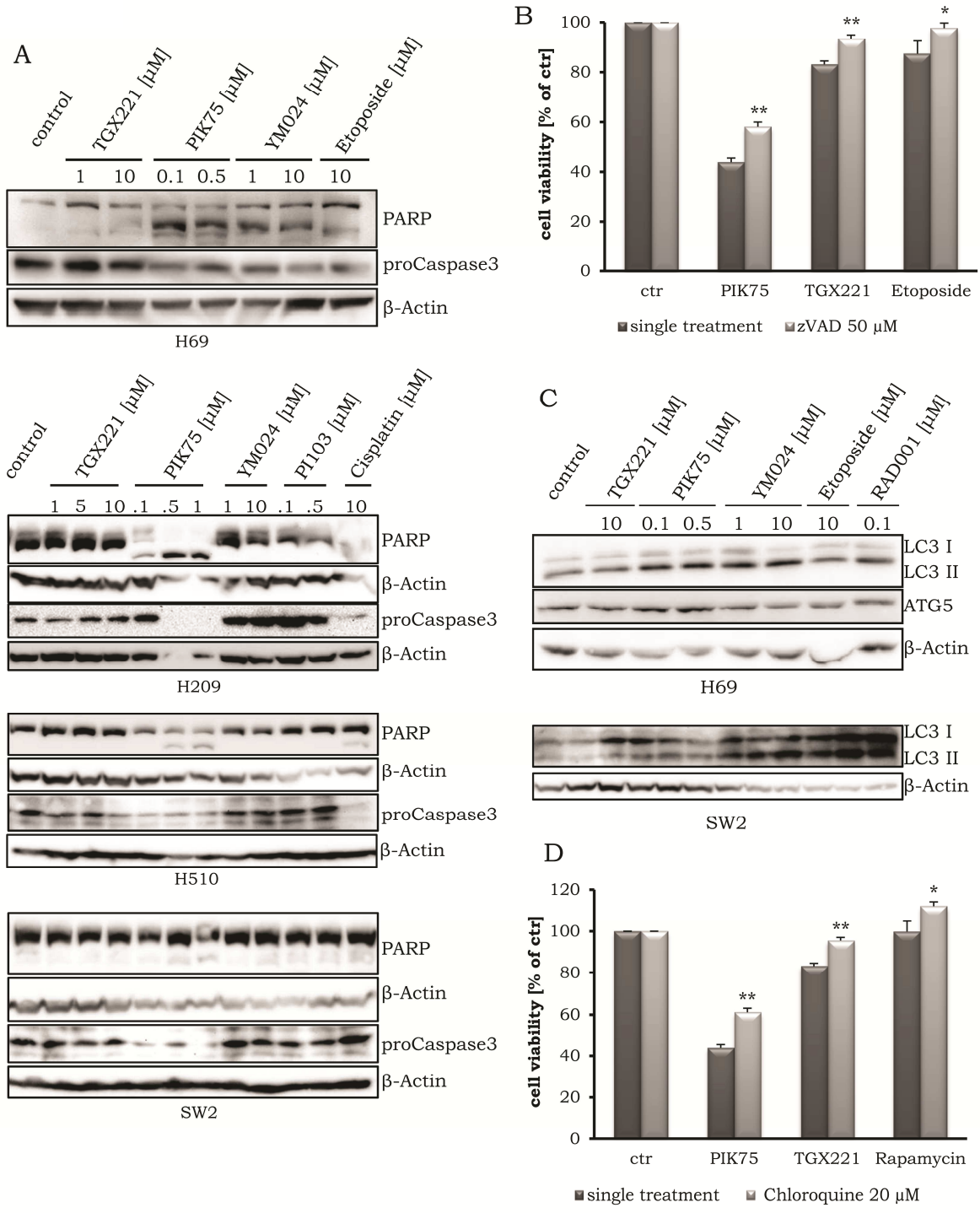


Figure 11 PI3K inhibition increases apoptosis and autophagy in SCLC. (a+c) H69, H209, H510 and SW2 cells grown in serum-containing medium were incubated with increasing concentrations of the PI3K p110α inhibitors PIK75 and YM024, the PI3K p110β inhibitor TGX221, RAD001 or etoposide/cisplatin. After 24 hours the cells were harvested and cell lysates analyzed by SDS-PAGE and Western blotting for the proteins indicated. (b+d) H69 cells grown in serum-containing medium were incubated with the PI3K inhibitors PIK75 (0.05 µM) and TGX221 (10 µM), rapamycin (0.1 µg/ml) or etoposide (10 µM) in absence or presence of zVAD-FMK (50 µM) or chloroquine (20 µM). Cell proliferation was assessed using the MTS assay after 12h. The data are mean of four replicates and three independently performed experiments. ** $p<0.01$, * $p<0.05$.

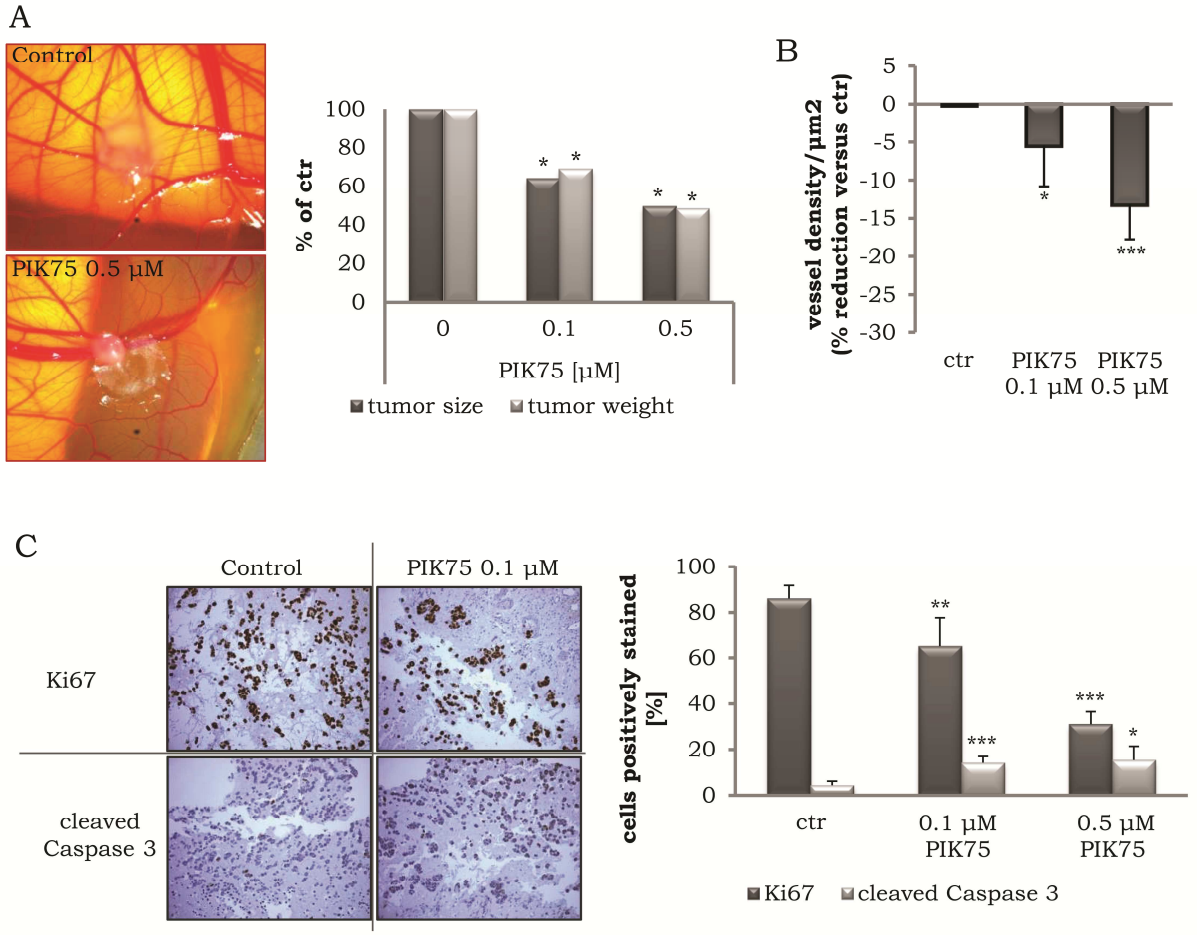


Figure 12 PI3K inhibition reduces tumor formation and vascularization *in vivo*. (a) H69 cells were treated with vehicle or different concentrations of PIK75 and applied on the CAM of chick embryos. On day 13 pictures and tumors were taken to analyze tumor size, weight (a) and vessel density (b). (b) Analysis of the vessel density reduction upon PI3P p110α inhibition in vehicle- or PIK75-treated H69 tumors (c) Immunostaining with Ki67 and cleaved caspase 3 comparing the expression in vehicle- and PIK75-treated SCLC tumor sections. (d) Analysis of Ki67 or caspase 3 positively stained tumor cells comparing vehicle- and PIK75-treated H69 tumor sections. (positively stained cells [%] = number of positive cells/total number of cells x 100). *** $p<0.001$, ** $p<0.01$, * $p<0.05$.

DNA microarray analysis of SCLC cells treated with isoform-specific PI3K inhibitors

In order to investigate whether the class I_A PI3K isoform p110α controls the expression of specific gene subsets in SCLC, we performed DNA microarray analysis in H69 cells treated with either vehicle, PIK75 targeting p110α, or TGX221 targeting p110β (Fig. 13A). The efficacy of the down-regulation of the Akt/mTOR by the respective inhibitors was demonstrated by Western blot analysis, as well as quantitative RT-PCR for VEGFA expression (Fig. 9C; Suppl. Fig. 1B+3). Inhibiting p110α significantly affected the expression of 3411 genes (P-value 0.01; FC ≥ 1.5), while inhibiting p110β resulted in significant changes (P-value 0.01; FC ≥ 1.5) in 4 genes, suggesting an important role of p110α but not p110β for the regulation of the expression of a subset of genes in SCLC (Fig. 13A). We next sought to investigate which genes were selectively affected by p110α inhibition, in comparison to p110β. Amongst these genes, in particular anti-apoptotic proteins of the Bcl-2 family of proteins were

found to be more significantly down-regulated in SCLC cells treated with p110α inhibitor, than in the case of the p110β inhibitor (Table 1).

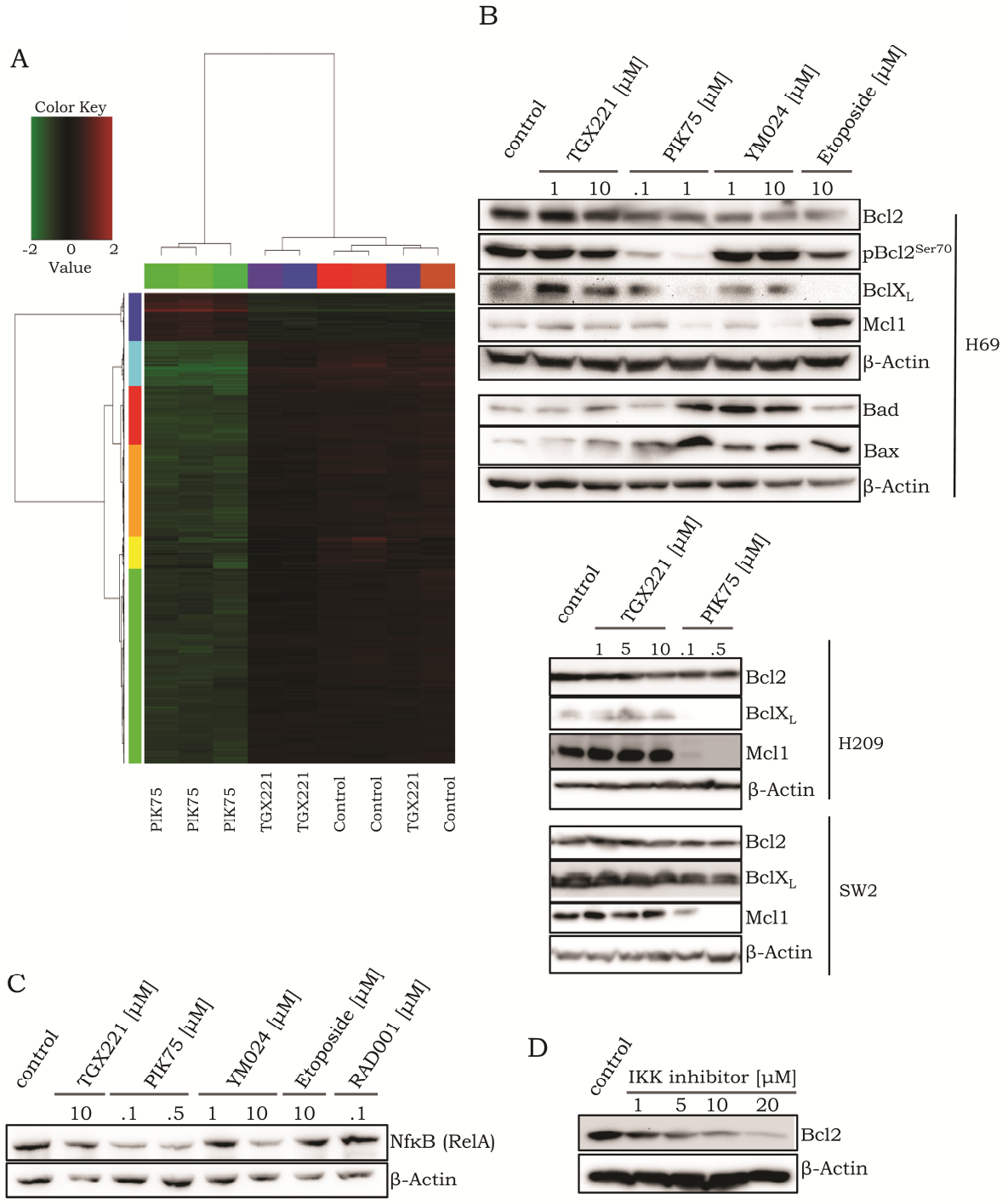


Fig. 13 The proteins of the Bcl2 family are downstream targets of p110α. (a) Gene expression analysis by DNA microarray: Heatmap of gene expression changes caused by p110α inhibition. (b+c+d) H69, H209 and SW2 (b) or H69 (c+d) cells grown in serum-containing medium were incubated with increasing concentrations of the PI3K p110α inhibitors PIK75 and YM024, the PI3K p110β inhibitor TGX221, RAD001 and etoposide/cisplatin or the IKK inhibitor wedelolactone (d). After 24 hours the cells were harvested and cell lysates analyzed by SDS-PAGE and Western blotting for the proteins indicated.

Table 1 Expression of proteins of the Bcl2-family and Bcl2-related proteins obtained by DNA microarray analysis of H69 cells treated with PIK75 versus control

Gene name	*Description*	*FC*	*P-value*
BCL2	*B-cell CLL/lymphoma 2*	*-1.9*	*0.0001*
BCL2L1	*BCL2-like 1*	*-1.3*	*0.0063*
MCL1	*myeloid cell leukemia sequence 1 (BCL2-related)*	*-1.8*	*0.0193*
BCL2L2	*BCL2-like 2*	*-1.9*	*0.0000*
BCL2L10	*BCL2-like 10 (apoptosis facilitator)*	*1.2*	*0.0099*
BCL2A1	*BCL2-related protein A1*	*1.2*	*0.0054*
BAX	*BCL2-associated X protein*	*-1.3*	*0.0095*
BAK1	*BCL2-antagonist/killer 1*	*1.1*	*0.1298*
BAD	*BCL2-associated agonist of cell death*	*1.2*	*0.0070*
BOK	*BCL2-related ovarian killer*	*1.2*	*0.0342*
BID	*BH3 interacting domain death agonist*	*-1.6*	*0.0002*
HRK	*harakiri, BCL2 interacting protein (contains only BH3 domain)*	*1.1*	*0.0095*
BCL2L11	*BCL2-like 11 (apoptosis facilitator)*	*1.4*	*0.0015*
BNIP1	*BCL2/adenovirus E1B 19kDa interacting protein 1*	*-1.0*	*0.2678*
BNIP2	*BCL2/adenovirus E1B 19kDa interacting protein 2*	*-1.1*	*0.3081*
BNIP3	*BCL2/adenovirus E1B 19kDa interacting protein 3*	*-2.9*	*0.0060*
BNIP3L	*BCL2/adenovirus E1B 19kDa interacting protein 3-like*	*-5.2*	*0.0024*
BNIPL	*BCL2/adenovirus E1B 19kD interacting protein like*	*1.2*	*0.0756*
BIK	*BCL2-interacting killer (apoptosis-inducing)*	*-1.4*	*0.0322*
BLK	*B lymphoid tyrosine kinase*	*1.3*	*0.0047*
PMAIP1	*phorbol-12-myristate-13-acetate-induced protein 1*	*1.4*	*0.0110*
BMF	*Bcl2 modifying factor*	*-1.1*	*0.2417*
BBC3	*BCL2 binding component 3*	*1.0*	*0.3285*
BCLAF1	*BCL2 associated transcription factor 1*	*-2.4*	*0.0017*
BCL2L13	*BCL2-like 13 (apoptosis facilitator)*	*-1.4*	*0.0028*
BCL2L14	*BCL2-like 14 (apoptosis facilitator)*	*1.2*	*0.0311*
BCL2L15	*BCL2-like 15*	*1.2*	*0.0026*
MOAP1	*modulator of apoptosis 1*	*-1.1*	*0.0403*
BCL2L12	*BCL2-like 12 (proline rich)*	*1.2*	*0.0052*
BAG1	*BCL2-associated athanogene*	*1.2*	*0.0144*
BAG2	*BCL2-associated athanogene 2*	*-1.1*	*0.1835*
BAG3	*BCL2-associated athanogene 3*	*1.4*	*0.0010*
BAG4	*BCL2-associated athanogene 4*	*1.1*	*0.4007*
BAG5	*BCL2-associated athanogene 5*	*1.3*	*0.0014*

The proteins of the Bcl-2 family are downstream targets of p110α

In order to validate the Bcl-2 family proteins as targets of p110α in SCLC cell lines, we used antibody arrays and Western blot analysis to confirm the results obtained from the DNA microarrays. A commercially available phosphorylation-antibody array revealed beside an impaired Akt pathway activation a down-regulation of anti-apoptotic Bcl2-family proteins (Suppl. Fig 5). Down-regulation of the expression and impaired activation of the anti-apoptotic Bcl2 family proteins could indeed be demonstrated at the protein level upon p110α inhibition (Fig. 13B). As it could be already observed before in the apoptotic response, the SCLC cell lines under study responded individually to the p110α

inhibition. In particular the cell lines H69 and H209 showed a strongly decreased expression of the Bcl-2 family members Bcl-2, Bcl-X_L and Mcl-1, whereas SW2 cells seemed to be more resistant. In addition, p110α inhibition induced the levels of the pro-apoptotic family members Bad and Bax (Fig. 13B). In contrast to the inhibitors of p110β, TGX221 had little effect on the expression levels of Bcl-2 family proteins (Fig. 13B). The anti-apoptotic Bcl2-family proteins Bcl2 and $BclX_L$ are targets of the transcriptional network of the transcription factor NFκB, which is controlling differential cell responses including immune response, cell proliferation and survival (301). To gain insight into the transcriptional networks affected by silencing of p110α in SCLC cells, we performed a biostatistics analysis of the gene expression data, using GeneGo. The transcriptional networks that were most significantly altered comprised HNF4α, SP1 and c-Myc, the estrogen receptor (ER) and also NFκB (Suppl. Table 1). By Western blot analysis of a whole cell lysate of H69 cells treated with PIK75 and YM024 targeting p110α we could observe deregulation of the NFκB expression, whereas p110β did not have any effect (Fig. 13C). These results suggested NFκB to be a downstream target of PI3K p110α signaling in SCLC and we assumed that its deregulation may have also an effect on the expression of the anti-apoptotic proteins Bcl2 and $BclX_L$. This could be confirmed by a decreased expression level of Bcl-2 in H69 cells treated with wedelolactone, an inhibitor of the IκB-kinase (IKK) (Fig. 13D).

Discussion

The PI3K/Akt/mTOR pathway has been demonstrated to play a key role in SCLC cell proliferation, survival, chemoresistance and migration. Mutations in *PIK3CA* and gene amplification were reported in primary SCLC, as well as increased expression of *PIK3CA* at the mRNA and protein level. Broad specificity PI3K/mTOR inhibitors have shown anti-tumor activity in SCLC models *in vitro* and *in vivo* (118). In this report, we have investigated the impact of isoform-specific inhibitors and RNAi targeting class I_A PI3K isoforms on SCLC cell responses. In general, agents targeting p110α reduced SCLC cell proliferation *in vitro*, impaired the SCLC tumor formation, which was accompanied by affected vascularization *in vivo*, and decreased the activation status of classical PI3K downstream targets, such as Akt, mTOR and S6K. Targeting p110α by RNAi or isoform-specific inhibitors had more pronounced effects on SCLC cell responses than in the case of p110β or p110δ, indicating a selective role for p110α in SCLC. This hypothesis was also confirmed by the observation that p110α was over-expressed in advanced stage SCLC, which was not the case for p110β. In view of these observations, we hypothesized that p110α may control the expression of a selective subset of genes implicated in SCLC cell proliferation and/or survival. The comparative DNA microarray analysis of SCLC cell lines in which either p110α or p110β were inhibited by selective compounds identified such a gene subset. The Bcl-2 family of proteins was validated as a downstream target of p110α by a combination of approaches. The observation that Bcl-2 expression was elevated in primary SCLC, in

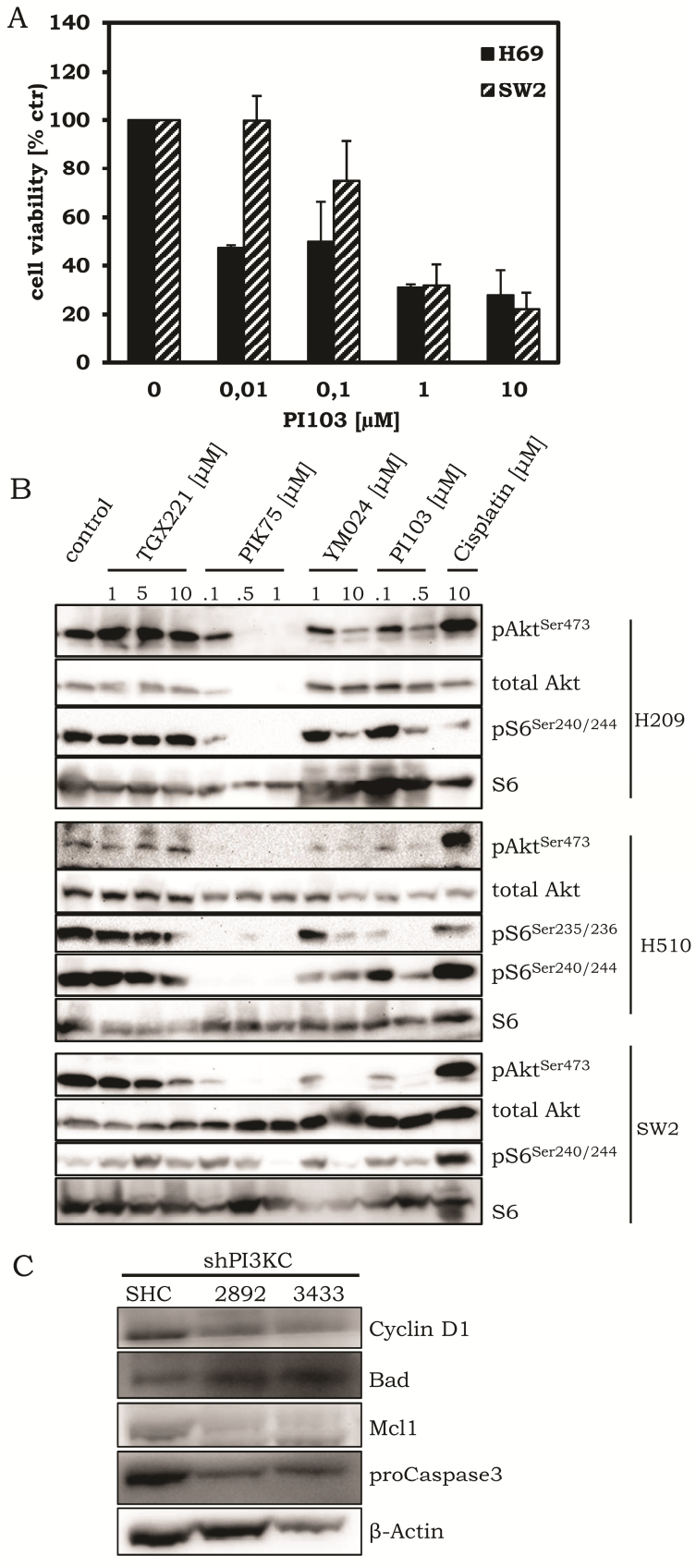
comparison to normal lung tissue further supports this model, in view of the over-expression of p110α. Importantly, the Bcl-2 family of proteins has been previously shown to play a crucial role in the survival of SCLC cell lines *in vitro* and *in vivo* (149, 160, 302-305). The p110α inhibitors induced increases in both SCLC apoptosis and autophagy, which is consistent with Bcl-2 family proteins being a target of p110α. Bcl-2 family proteins are key regulator of both apoptosis and autophagy (306) and their reduction via the inhibition of the p110α/NFκB pathway may play an essential role in the effects of the p110α inhibitors in SCLC.

We have previously evaluated the mTOR inhibitor everolimus in SCLC cell lines and found that it was effective in a subset of SCLC cell lines characterized by activation of the Akt/mTOR pathway and low expression levels of anti-apoptotic Bcl-2 family proteins (304). In view of the results obtained with isoform-selective inhibitors of p110α, it can be speculated that these agents may be more potent, since they induce a down-regulation of anti-apoptotic Bcl-2 family proteins and of the activity of the Akt/mTOR pathway. This may be particularly relevant for SCLC tumors bearing activating mutations in the *PIK3CA* gene.

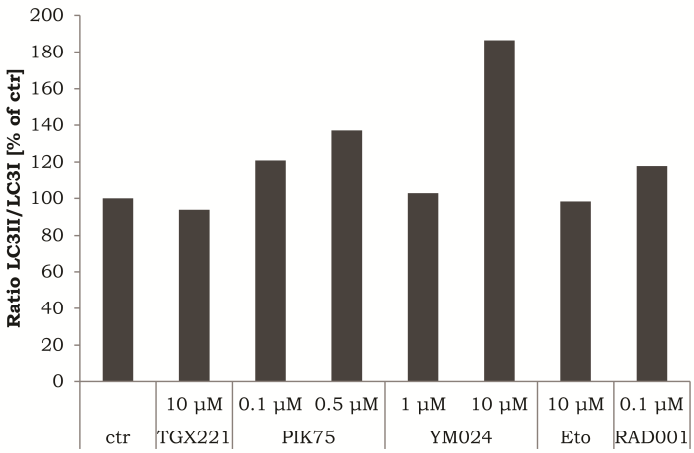
Interestingly, our previous work in neuroblastoma has shown that the class I_A PI3K isoform p110δ contributes to cell proliferation and survival by controlling the activation of the mTOR pathway and the expression levels of anti-apoptotic Bcl-2 family proteins. Over-expression of p110δ was found in a subset of primary neuroblastoma and cell lines, and p110δ was essential for signal transduction by receptor tyrosine kinases, such as the IGF-1R and EGFR (277). In contrast, in SCLC cell lines, p110α appears to play a more important role in the activation of the Akt/mTOR pathway, which may explain the impact of agents targeting this isoform on SCLC proliferation and survival. A previous report has shown that the activity of any class I_A PI3K isoform can maintain cell survival (307). Therefore, the relative importance of class I_A PI3K isoforms in selected cancer types may be, in part, attributed to differences in expression levels.

Thus, targeting PI3K p110α signaling may represent an attractive novel approach to develop novel therapies for SCLC. Indeed, there now exist different pharmacological inhibitors of this isoform, which will soon enter clinical trials in oncology, and could, in the future, be developed as new drugs for SCLC.

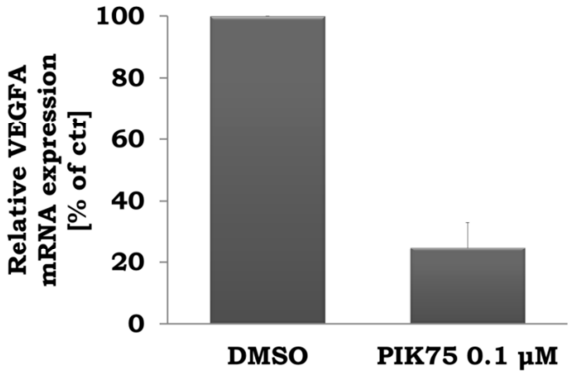
Supplementary Data



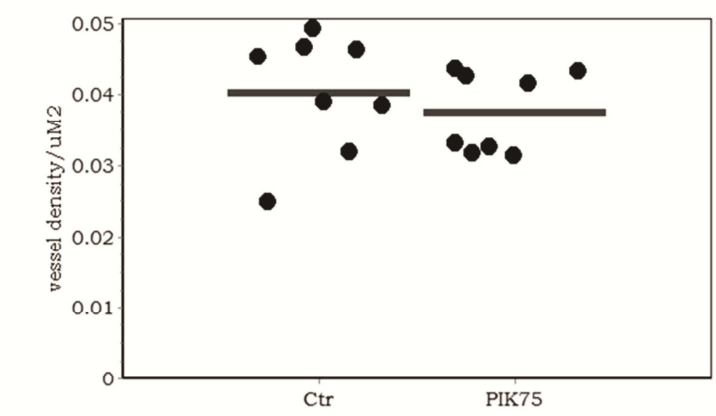
Supplementary Fig. 1. PI3K inhibition blocks SCLC cell viability and PI3K downstream signaling. (a) The SCLC cell lines H69 and SW2 were incubated with increasing concentrations of the dual PI3K p110α/mTOR inhibitor PI103. Cell viability was assessed using the MTS assay after 3 days. (b) H209, H510 and SW2 SCLC cells were incubated with increasing concentrations of the PI3K p110α inhibitors PIK75, YM024, and PI103 (PI3K p110α/mTOR inhibitor) and the PI3K p110β inhibitor TGX221, and cisplatin. After 24 hours the cells were harvested and whole cell lysates analyzed by SDS-PAGE and Western blotting for the proteins indicated. (c) **Stable Silencing of p110α affects cell viability and PI3K downstream signaling activation.** In H69 cells p110α was stably silenced by the lentiviral delivery of shRNA constructs. Cell lysates were analyzed by SDS-PAGE and Western blotting with antibodies for the proteins indicated.



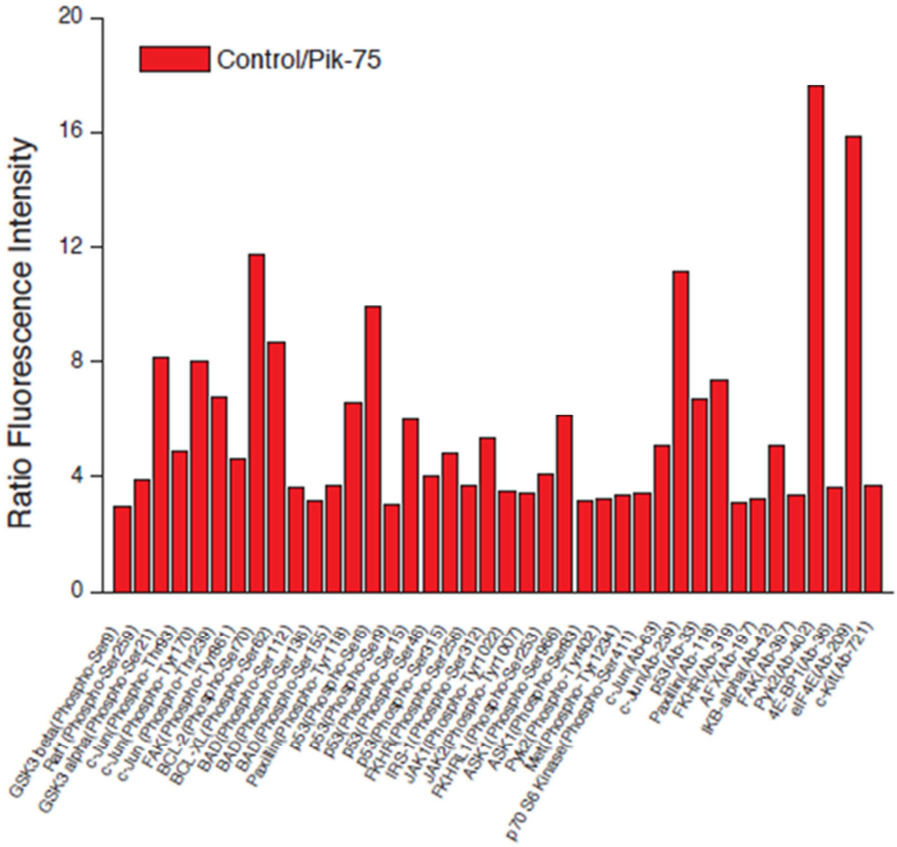
Supplementary Fig. 2. PI3K inhibition increases autophagy in SCLC. Analysis of the ratio of LC3II/LC3I. H69 cells grown in serum-containing medium were incubated with increasing concentrations of the PI3K p110α inhibitors PIK75 and YM024, the PI3K p110β inhibitor TGX221, RAD001 or etoposide/cisplatin.



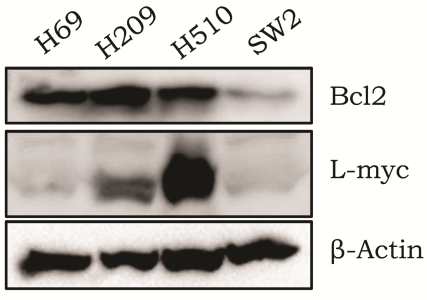
Supplementary Fig. 3. PI3K p110α inhibition decreases VEGFA in SCLC. Analysis of the relative VEGFA mRNA expression in H69 cells grown in serum-containing medium treated with the PI3K p110α inhibitor PIK75.



Supplementary Fig. 4. PI3K inhibition reduces vascularization *in vivo*. As negative control Matrigel with vehicle or PIK75 (0.5 µM) was applied on the CAM of chick embryos. On day 13 pictures were taken to analyze the vessel density.



Supplementary Fig. 5 Antibody array analysis of PIK75 treated H69 cells. Qualitative ratio of the expression/phosphorylation of the indicated proteins of H69 cells treated with 0.1 µM PIK75 or DMSO. The effect of the PI3K inhibitor PIK75 on the phosphorylation and expression of proteins of the PI3K/Akt pathway was assessed by employing a commercially available phospho-antibody array slides containing proteins of the PI3K/Akt pathway (Phospho-Akt Antibody Array PAA137; Fullmoon Biotechnology). Briefly, H69 cells were treated with 0.1 µM PIK75 for 24h and the antibody array slides were pre-treated and then incubated with the biotinylized whole cell protein lysate according to the recommended protocol with minor changes by using an automated hybridization station HS4800.



Supplementary Fig. 6 Expression of Bcl2 and L-myc in SCLC cell lines. Whole cell lysates of the SCLC cell lines H69, H209, H510 and SW2 were analyzed by SDS-PAGE and Western blotting with antibodies for the proteins Bcl2 and L-myc. The expression of β-Actin was used as internal control.

Suppl. Table 1. Biostatistical analysis of the transcriptional networks impaired by p110α inhibition

No	*Network*	*GO Processes*	*p-Value*	*zScore*	*gScore*
1	SP1	cellular process (92.5%; 1.476e-20), developmental process (49.8%; 2.302e-20), anatomical structure development (44.4%; 5.950e-19), metabolic process (73.0%; 5.470e-18), cellular component organization (42.3%; 6.564e-17)	0.000E+00	100.52	100.52
2	HNF4-alpha	cellular metabolic process (68.4%; 1.211e-20), primary metabolic process (66.7%; 2.208e-18), metabolic process (72.6%; 4.695e-17), cellular process (89.3%; 7.217e-15), cellular macromolecule metabolic process (50.0%; 8.455e-14)	0.000E+00	99.89	99.89
3	c-Myc	cellular metabolic process (71.1%; 1.934e-23), primary metabolic process (70.2%; 4.226e-22), cellular nitrogen compound metabolic process (50.7%; 1.487e-20), metabolic process (75.6%; 5.741e-20), nucleobase, nucleoside, nucleotide and nucleic acid metabolic process (47.1%; 9.153e-20)	0.000E+00	97.11	97.11
4	ESR1 (nuclear)	developmental process (48.2%; 2.340e-11), positive regulation of cellular process (37.6%; 3.012e-11), positive regulation of metabolic process (27.0%; 3.968e-11), response to estrogen stimulus (11.3%; 4.872e-11), positive regulation of macromolecule metabolic process (25.5%; 5.365e-11)	8.740E-237	77.36	77.36
5	p53	regulation of metabolic process (56.6%; 1.172e-13), regulation of primary metabolic process (51.9%; 1.489e-13), regulation of macromolecule metabolic process (49.6%; 2.922e-13), regulation of cellular metabolic process (51.2%; 7.187e-13), cellular macromolecule metabolic process (54.3%; 7.250e-11)	4.920E-215	73.75	73.75
6	CREB1	regulation of metabolic process (57.8%; 1.726e-14), regulation of primary metabolic process (52.3%; 9.134e-14), regulation of macromolecule metabolic process (49.2%; 7.076e-13), regulation of cellular metabolic process (50.8%; 1.666e-12), regulation of cellular process (75.0%; 2.980e-12)	2.300E-213	73.47	73.47
7	EGR1	regulation of macromolecule metabolic process (55.4%; 8.872e-16), cellular macromolecule metabolic process (62.5%; 5.353e-15), macromolecule metabolic process (65.2%; 2.335e-14), cellular metabolic process (73.2%; 7.536e-14), regulation of primary metabolic process (54.5%; 1.119e-13)	2.330E-183	68.14	68.14
8	HIF1A	cellular macromolecule metabolic process (69.0%; 3.345e-18), macromolecule metabolic process (70.0%; 2.755e-16), metabolic process (85.0%; 3.678e-16), cellular metabolic process (78.0%; 7.322e-16), protein modification process (40.0%; 9.638e-16)	4.410E-165	64.67	64.67
9	Androgen receptor	primary metabolic process (77.0%; 4.894e-15), macromolecule metabolic process (67.0%; 3.589e-14), protein modification by small protein conjugation (18.0%; 4.469e-14), cellular macromolecule metabolic process (63.0%; 7.110e-14), metabolic process (82.0%; 7.895e-14)	2.010E-163	64.34	64.34
10	NF-kB	protein modification by small protein conjugation (19.2%; 2.513e-15), protein ubiquitination (18.2%; 1.318e-14), protein modification by small protein conjugation or removal (19.2%; 4.673e-14), protein modification process (37.4%; 1.420e-13), macromolecule modification (38.4%; 1.604e-13)	9.170E-162	64.02	64.02

Suppl. Table 2 Genetic alterations and expression profile of the PI3K p110α and the tumor suppressors PTEN, TP53, and RB in 3 SCLC cell lines

	PIK3CA	*PTEN*	*TP53*	*RB*
H69	*activating mutation (1)* *gene copy number 3.69 (6)* *phenotype: over-expressed protein (9)*	*wt (geno-/phenotype) (1)*	*inactivating mutation: glu->stop (2)* *phenotype: reduced TP53 protein expression (7)*	*inactivating genetic alterations (3) (4) (5)* *phenotype: reduced RB protein expression (8)*
H209	*genotype wt (6)* *gene copy number 1.49 (6)* *phenotype wt (compared to normal pneumocytes) (9)*	*phenotype wt (compared to normal pneumocytes) (9)*	*point mutation causing in disrupted splice site in* TP53 (10)	*RB1 C706F mutation (10)* *phenotype: abberant RB protein expression (3, 11)*
H510	*WT (6)* *gene copy number 3.17 (6)* *phenotype wt (compared to normal pneumocytes) (9)*	*phenotype wt (compared to normal pneumocytes) (9)*	*inactivating mutation: arg->gly (12)*	*phenotype: absent RB protein (13) (11)*

References
1. Shibata, T., Kokubu, A., Tsuta, K., and Hirohashi, S. Oncogenic mutation of PIK3CA in small cell lung carcinoma: a potential therapeutic target pathway for chemotherapy-resistant lung cancer. Cancer Lett, 283: 203-211, 2009.
2. Hensel, C. H., Xiang, R. H., Sakaguchi, A. Y., and Naylor, S. L. Use of the single strand conformation polymorphism technique and PCR to detect p53 gene mutations in small cell lung cancer. Oncogene, 6: 1067-1071, 1991.
3. Hensel, C. H., Hsieh, C. L., Gazdar, A. F., Johnson, B. E., Sakaguchi, A. Y., Naylor, S. L., Lee, W. H., and Lee, E. Y. Altered structure and expression of the human retinoblastoma susceptibility gene in small cell lung cancer. Cancer Res, 50: 3067-3072, 1990.
4. Harbour, J. W., Lai, S. L., Whang-Peng, J., Gazdar, A. F., Minna, J. D., and Kaye, F. J. Abnormalities in structure and expression of the human retinoblastoma gene in SCLC. Science, 241: 353-357, 1988.
5. Yokota, J., Akiyama, T., Fung, Y. K., Benedict, W. F., Namba, Y., Hanaoka, M., Wada, M., Terasaki, T., Shimosato, Y., Sugimura, T., and et al. Altered expression of the retinoblastoma (RB) gene in small-cell carcinoma of the lung. Oncogene, 3: 471-475, 1988.
6. Yamamoto, H., Shigematsu, H., Nomura, M., Lockwood, W. W., Sato, M., Okumura, N., Soh, J., Suzuki, M., Wistuba, II, Fong, K. M., Lee, H., Toyooka, S., Date, H., Lam, W. L., Minna, J. D., and Gazdar, A. F. PIK3CA mutations and copy number gains in human lung cancers. Cancer Res, 68: 6913-6921, 2008.
7. Han, J. Y., Chung, Y. J., Park, S. W., Kim, J. S., Rhyu, M. G., Kim, H. K., and Lee, K. S. The relationship between cisplatin-induced apoptosis and p53, bcl-2 and bax expression in human lung cancer cells. Korean J Intern Med, 14: 42-52, 1999.
8. Rygaard, K., Sorenson, G. D., Pettengill, O. S., Cate, C. C., and Spang-Thomsen, M. Abnormalities in structure and expression of the retinoblastoma gene in small cell lung cancer cell lines and xenografts in nude mice. Cancer Res, 50: 5312-5317, 1990.
9. Arcaro, A., Khanzada, U. K., Vanhaesebroeck, B., Tetley, T. D., Waterfield, M. D., and Seckl, M. J. Two distinct phosphoinositide 3-kinases mediate polypeptide growth factor-stimulated PKB activation. EMBO J, 21: 5097-5108, 2002.
10. Pleasance, E. D., Stephens, P. J., O'Meara, S., McBride, D. J., Meynert, A., Jones, D., Lin, M. L., Beare, D., Lau, K. W., Greenman, C., Varela, I., Nik-Zainal, S., Davies, H. R., Ordonez, G. R., Mudie, L. J., Latimer, C., Edkins, S., Stebbings, L., Chen, L., Jia, M., Leroy, C., Marshall, J., Menzies, A., Butler, A., Teague, J. W., Mangion, J., Sun, Y. A., McLaughlin, S. F., Peckham, H. E., Tsung, E. F., Costa, G. L., Lee, C. C., Minna, J. D., Gazdar, A., Birney, E., Rhodes, M. D., McKernan, K. J., Stratton, M. R., Futreal, P. A., and Campbell, P. J. A small-cell lung cancer genome with complex signatures of tobacco exposure. Nature, 463: 184-190, 2010.
11. Modi, S., Kubo, A., Oie, H., Coxon, A. B., Rehmatulla, A., and Kaye, F. J. Protein expression of the RB-related gene family and SV40 large T antigen in mesothelioma and lung cancer. Oncogene, 19: 4632-4639, 2000.
12. D'Amico, D., Carbone, D., Mitsudomi, T., Nau, M., Fedorko, J., Russell, E., Johnson, B., Buchhagen, D., Bodner, S., Phelps, R., and et al. High frequency of somatically acquired p53 mutations in small-cell lung cancer cell lines and tumors. Oncogene, 7: 339-346, 1992.
13. Horowitz, J. M., Park, S. H., Bogenmann, E., Cheng, J. C., Yandell, D. W., Kaye, F. J., Minna, J. D., Dryja, T. P., and Weinberg, R. A. Frequent inactivation of the retinoblastoma anti-oncogene is restricted to a subset of human tumor cells. Proc Natl Acad Sci U S A, 87: 2775-2779, 1990.

Suppl. Table 3
Expression profile of the proliferation marker Ki67 in 4 SCLC cell lines

SCLC cell line	(mean of % positive cells, n=3 samples
H69	77.3%
H209	72.3%
H510	45.3%
SW2	54.6%

NOVEL AGENTS TARGETING THE IGF-IR/PI3K PATHWAY IMPAIR CELL PROLIFERATION AND SURVIVAL IN SUBSETS OF MEDULLOBLASTOMA AND NEUROBLASTOMA

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Grant Support: European Union FP7 (ASSET, project number: 259348), Gottfried und Julia Bangerter-Rhyner-Stiftung, Schüller Stiftung, Frieda Locher-Hofmann-Stiftung, Huggenberger-Bischoff Stiftung zur Krebsforschung, Jubiläumsstiftung der Schweizerischen Mobiliar Genossenschaft, Fondation FORCE, Department of Clinical Research of the University of Bern

Running title: IGF-IR targeting in medulloblastoma and neuroblastoma

Keywords: apoptosis; IGF-IR; neuroblastoma; medulloblastoma; PI3K; proliferation

This manuscript has been submitted to the journal PLoS ONE and is currently *in press*.

Abstract

The receptor tyrosine kinase (RTK)/phosphoinositide 3-kinase (PI3K) pathway is fundamental for cancer cell proliferation and is known to be frequently altered and activated in neoplasia, including embryonal tumors. Based on the high frequency of alterations, targeting components of the PI3K signaling pathway is considered to be a promising therapeutic approach for cancer treatment. Here, we have investigated the potential of targeting the axis of the insulin-like growth factor-1 receptor (IGF-1R) and PI3K signaling in two common cancers of childhood: neuroblastoma, the most common extracranial tumor in children and medulloblastoma, the most frequent malignant childhood brain tumor. By treating neuroblastoma and medulloblastoma cells with R1507, a specific humanized monoclonal antibody against the IGF-1R, we could observe cell line-specific responses and in some cases a strong decrease in cell proliferation. In contrast, targeting the PI3K p110α with the specific inhibitor PIK75 resulted in broad anti-proliferative effects in a panel of neuro- and medulloblastoma cell lines. Additionally, sensitization to commonly used chemotherapeutic agents occurred in neuroblastoma cells upon treatment with R1507 or PIK75. Furthermore, by studying the expression and phosphorylation state of IGF-1R/PI3K downstream signaling targets we found down-regulated signaling pathway activation. In addition, apoptosis occurred in embryonal tumor cells after treatment with PIK75 or R1507. Together, our studies demonstrate the potential of targeting the IGF-1R/PI3K signaling axis in embryonal tumors. Hopefully, this knowledge will contribute to the development of urgently required new targeted therapies for embryonal tumors.

Introduction

Second to accidents, cancer is still the leading cause of death for children. Embryonal tumors represent approximately 30% of childhood malignancies and often display resistance to current therapeutic regimens. Therefore, embryonal tumors are associated with lower survival rates compared to other childhood cancers**.** Treatment failure for disseminated disease is frequent, and results in survival rates < 20%. Thus, novel therapeutic options are urgently needed for this group of tumors to improve survival rates and quality of life of patients. Embryonal tumors are dysontogenetic tumors whose pathological features resemble those of the developing organ or tissue of origin and include the entities medulloblastoma and neuroblastoma. Medulloblastoma is the most common malignant brain tumor in children and accounts for approximately 20% to 25% of all pediatric central nervous system tumors. Neuroblastoma is an embryonal tumor that originates from developing neural crest tissues. It is the most common extracranial solid tumor and is responsible for 15% of all cancer-related deaths in childhood. The fact that these cancers occur in infants and young children suggests that only a limited number of genetic changes may lead to tumor development, making these cancers an attractive model to identify new molecular targets. The development of novel targeted therapies is of particular importance for embryonal tumors, as these malignancies are orphan diseases. Common intracellular signaling pathways and chromosomal deletions including 1p36 and 11q loss have been previously identified in different embryonal tumors, including medulloblastoma and neuroblastoma (308-317).

Several intracellular signaling pathways have indeed been demonstrated to play a key role in embryonal tumor biology. Indeed, polypeptide growth factors such as insulin-like growth factor-1 (IGF-1), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), neuregulins and neurotrophins have been shown to control embryonal tumor proliferation, survival, differentiation and metastasis (272, 318-321) by binding to specific receptor tyrosine kinases (RTKs). Moreover, expression of the ErbB-2 and ErbB-4 RTKs in embryonal tumor samples was shown to correlate with reduced patient survival, while Trk receptor expression correlated with a less aggressive tumor phenotype (319). Therefore a better understanding of the involvement of RTKs and their downstream targets in human embryonal tumor biology may yield important clues for the development of new drugs for the disease. Targeting receptor tyrosine kinases such as the IGF-1R is a promising approach to develop novel anti-cancer therapies in embryonal tumors, such as neuroblastoma and sarcoma (321-329). Indeed the first results from clinical trials evaluating the safety and efficacy of IGF-1R neutralising antibodies in children and adolescents with embryonal tumors have been reported (330, 331). In these trials, the humanized IGF-1R neutralizing antibody R1507 displayed minimal toxicities and some responses in ESFT were observed (330, 331). Importantly, no dose-limiting toxicities were identified and the maximum tolerated dose was not reached (330). Human embryonal tumor cells have been reported to express a variety of growth factor receptors, some of which can be activated by mutations, over-expression and/or establishment of autocrine loops (319). Amongst these polypeptide growth factor receptors are the RTKs IGF-1R, EGFR, ALK, ErbB-2, ErbB-4, c-Kit, PDGFR, Trk and

fibroblast growth factor receptor (FGFR) (233, 234, 332-345). Therefore, given that embryonal tumor cells express a variety of different growth factor receptors, targeting individual receptors may not provide a successful therapeutic strategy in all embryonal tumor entities. A potentially complementary approach would be to identify signaling molecules which lie downstream of several different growth factor receptors and which are essential for transmitting their proliferative and/or survival message. Combinatorial targeting of receptor tyrosine kinases (such as the IGF-1R) and their downstream signaling mediators is a very promising approach to develop more efficient anti-cancer therapies (322, 323, 328, 346-348).

The phosphoinositide 3-kinase (PI3K) plays a crucial role in controlling cell proliferation, survival and motility/metastasis downstream of many different growth factor receptors and oncogenic Ras mutants (1, 349-351). PI3K signaling activates a crucial intracellular signaling pathway involving phosphoinositide-dependent protein kinase-1 (PDK1), Akt, the mammalian target of rapamycin (mTOR) and the ribosomal protein S6 kinase (S6K), which controls cell growth, proliferation and survival (1, 349, 350). The importance of PI3K/Akt/mTOR signaling in cancer is highlighted by the fact that mutations in the tumor suppressor gene *PTEN* occur frequently in human tumors, including glioblastoma (1, 352-354). PTEN is a phosphatase that antagonizes the action of PI3K by de-phosphorylating the D-3 position of poly-phosphoinositides (1, 352, 353). Reduced expression of PTEN resulting in activation of PI3K signaling was recently described in embryonal tumors such as medulloblastoma and neuroblastoma (286, 355). Moreover, various reports have described activating mutations in the *PIK3CA* gene encoding the catalytic p110□ isoform of PI3K in a variety of human cancers, including, breast, colon and ovarian cancer, as well as embryonal tumors (283, 354, 356). In addition, PI3K/Akt/mTOR signaling has been demonstrated to mediate the proliferation of embryonal tumor cells (357, 358) and to contribute to signaling by ErbB-2 and IGF-1R (281, 359, 360). Activation of Akt was also reported in embryonal tumors, correlating with poor outcome in some entities (280). Thus, targeting the PI3K/Akt/mTOR signaling pathway may represent an attractive novel approach to develop novel therapies for embryonal tumors (361). Indeed, there now exist multiple pharmacological inhibitors of the PI3K/Akt/mTOR pathway which have entered clinical trials for adult and pediatric cancer (292, 348, 349, 351, 362, 363). The PI3K/Akt/mTOR pathway is also an important contributor to the resistance of human tumors to drugs targeting receptor tyrosine kinases (364-366). Inhibitors of the PI3K/Akt/mTOR signaling pathway have also been shown to be effective in combination with IGF-1R inhibitors (326, 347).

In the present report, we have evaluated the anti-proliferative potential of the humanized anti-IGF-1R antibody R1507 and of PIK75, a class I_A PI3K inhibitor, in medulloblastoma and neuroblastoma cell lines. We present evidence that these agents are effective as monotherapies in subsets of embryonal tumor cell lines and can be effectively combined with standard chemotherapeutic drugs.

Materials and methods

Antibodies & Reagents

Antibodies specific for IGF1Rβ, PI3K p110α, Akt1/2/3, ERK 1/2, S6 protein, Caspase-3 (Santa Cruz Biotechnology, CA, USA), phospho-ERK1/2 (Thr^{202}/Tyr^{204}), phospho-Akt (Ser^{473}; Thr^{308}), poly (ADP-ribose) polymerase (PARP), S6 protein,phospho-S6 (Ser^{235}/Ser^{236}; Ser^{240}/Ser^{244}) p-IGF-1R(Cell Signaling Technology), β-actin (Sigma Aldrich).

The PI3K inhibitor PIK-75 (299) was dissolved in DMSO (Sigma, Buchs, Switzerland) at 10 mM and diluted into cell culture medium just before use. R1507, a fully human IgG1 monoclonal antibody to IGF-1R, was obtained from Roche, and was diluted directly into the medium immediately before use. The chemotherapeutic agents cisplatin (Bristol-Myers Squibb), doxorubicin (Pfizer) and etoposide (Calbiochem) were used in combination with PIK75 or R1507 at the indicated concentrations.

Cell lines, cell culture, cell proliferation and apoptosis

Human neuroblastoma cell lines were kindly provided by Dr. Brodeur, Children's Hospital of Philadelphia. The cells were grown in RPMI (Life Technologies/Invitrogen) supplemented with 10% (v/v) fetal calf serum (FCS) and penicillin/streptomycin/L-glutamine and passaged every 3-5 days by trypsinization (277, 326).

The medulloblastoma cell lines' provenience has been previously described. DAOY, UW-228 and PFSK human cell lines were purchased from the American Type Culture Collection. D341 Med and D458 Med medulloblastoma cells were the kind gift of Dr. Henry Friedman (Duke University, Durham, NC). Cell lines that were not purchased from the American Type Culture Collection in 2009 were tested for their authentication by karyotypic analysis using molecular cytogenetic techniques, such as comparative genomic hybridization. DAOY medulloblastoma cell line was grown in Richter's MEM Zinc option medium (Invitrogen) with 10% FCS (fetal bovine serum; Sigma) and penicillin/streptomycin (Invitrogen). PFSK primitive neuroectodermal tumor (PNET) cell line was grown in RPMI 1640 (Invitrogen) with 10% FCS and penicillin/streptomycin/L-glutamine. The UW-228 medulloblastoma cell line was grown in DMEM (Dulbecco's modified Eagle's medium; Invitrogen) with 10% FCS and penicillin/streptomycin/L-glutamine.D341 Med and D458 Med medulloblastoma cell lines were grown like DAOY but with the addition of 100M non-essential amino acids (GIPCO^{TM} MEM Invitrogen). All cells were grown in a humidified atmosphere at 37° and 5% CO_2 (284, 367).

For cell viability assays neuroblastoma and medulloblastoma cells were seeded in 96-well plates at a density of 3'000-10´000 cells/well and grown for 48-118 hrs in cell culture containing high (10%) serum. Cell proliferation was analyzed by the CellTiter 96$^{®}$AQ$_{ueous}$One Solution Cell Proliferation Assay (Promega) according to the manufacturer's instructions. For detection of apoptosis, NB and MB cells were seeded on 6-well plates and incubated for 24 h in the presence or absence of PIK75, R1507 or cisplatin. After lysis of the cells the protein samples were analyzed by SDS-PAGE and Western blot

with anti-PARP and anti-caspase-3 antibodies. Additionally, apoptosis was analyzed by caspase 3/7 activation using the Caspase-Glo 3/7 Assay (Promega), according to the manufacturer's instruction.

Western blotting

Cell lysates were prepared in RIPA buffer (50mM Tris-Cl, pH 6.8, 100mM NaCl, 1% Triton X-100, 0.1% SDS) supplemented with Complete Mini Protease Inhibitor Cocktail (Roche Applied Sciences) and with the phosphatase inhibitors β-glycerophosphate (20mM) and Na3VO4 (200 mM) and normalized using a bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA). Cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a hydrophobic polyvinylidenedifluoride (PVDF) membrane (Hybond-P; Amersham Biosciences,GE Healthcare, UK) and immunoblotted with the indicated antibodies prior to chemiluminescent detection (SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific Inc., Rockford, IL, USA).

Statistical analysis

Analysis of variance was used to assess statistical significance of differences between groups. p values <0.05 were considered as significant.

Results

Anti-proliferative activity of R1507 and PIK75 in panels of neuroblastoma and medulloblastoma cell lines

We have previously described panels of neuroblastoma and medulloblastoma cell lines, which were characterized for expression of components of the IGF-1R/PI3K signaling pathway (277, 284, 326). In the present study, the impact of the humanized anti-IGF-1R antibody R1507 was evaluated on cell proliferation *in vitro* in the panels of neuroblastoma and medulloblastoma cell lines (Fig. 14). The antibody displayed anti-proliferative activity in 2 out of 8 neuroblastoma cell lines, namely SH-SY5Y and LAN1 (Fig. 14A and 22A). In SH-SY5Y, R1507 induced a maximal decrease in cell viability of 60% at 12.5 µg/ml (Fig. 14A). In LAN1 a maximal activity of ~25% reduction in cell proliferation was observed (Fig. 22A). R1507 showed anti-proliferative activity in 2 out of 5 medulloblastoma cell lines, namely PFSK and D458 (Fig. 14B). In D458, a maximal decrease in cell proliferation (60%) was observed at 15 µg/ml, while in PFSK the maximal effect was 40% inhibition of the response (Fig. 14B). In NB and MB cell lines, the activity of R1507 was cell-line specific and the antibody had a profile similar to the IGF-1R tyrosine kinase inhibitor NVP-AEW541 (326). NVP-AEW541 was more active in SH-SY5Y and LAN-1 than in other neuroblastoma cell lines (326), and PFSK were more sensitive to NVP-AEW541 than DAOY and UW228 cells.

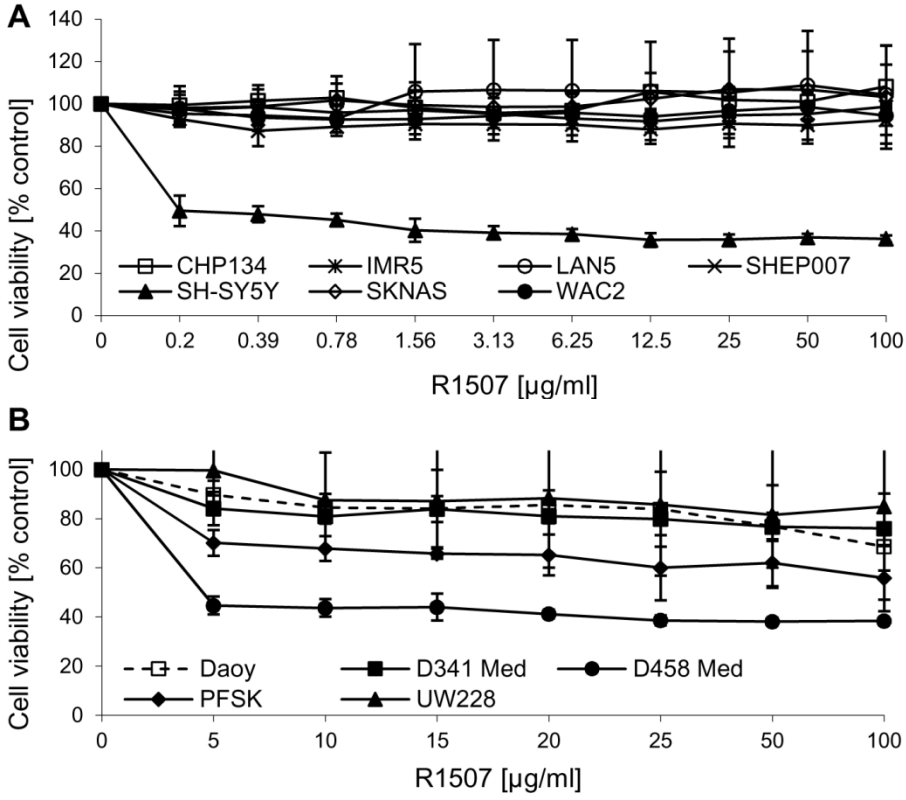


Figure 14. The effect of R1507 on cell proliferation of NB and MB cells. A panel of NB cell lines (A) and MB cell lines (B) were incubated with increasing concentrations of the antibody R1507 inhibiting the IGF-IR in serum-containing medium. Cell viability was assessed using the MTS assay after 2 days. The data represent the mean with SD from at least 6 replicates and 3 independent experiments.

The impact of the class I_A PI3K inhibitor PIK75 was evaluated on cell proliferation *in vitro* in the panels of neuroblastoma and medulloblastoma cell lines (Fig. 15). The inhibitor displayed strong anti-proliferative activity in the neuroblastoma cell line panel, with IC_{50} values in the range of 50-100 nM (Fig. 15A). In medulloblastoma cell lines, PIK75 was more active in D341 and D458 cells (IC_{50}= 20 nM) than in UW228 cells. The activity of PIK75 in DAOY and PFSK cells was previously described (284).

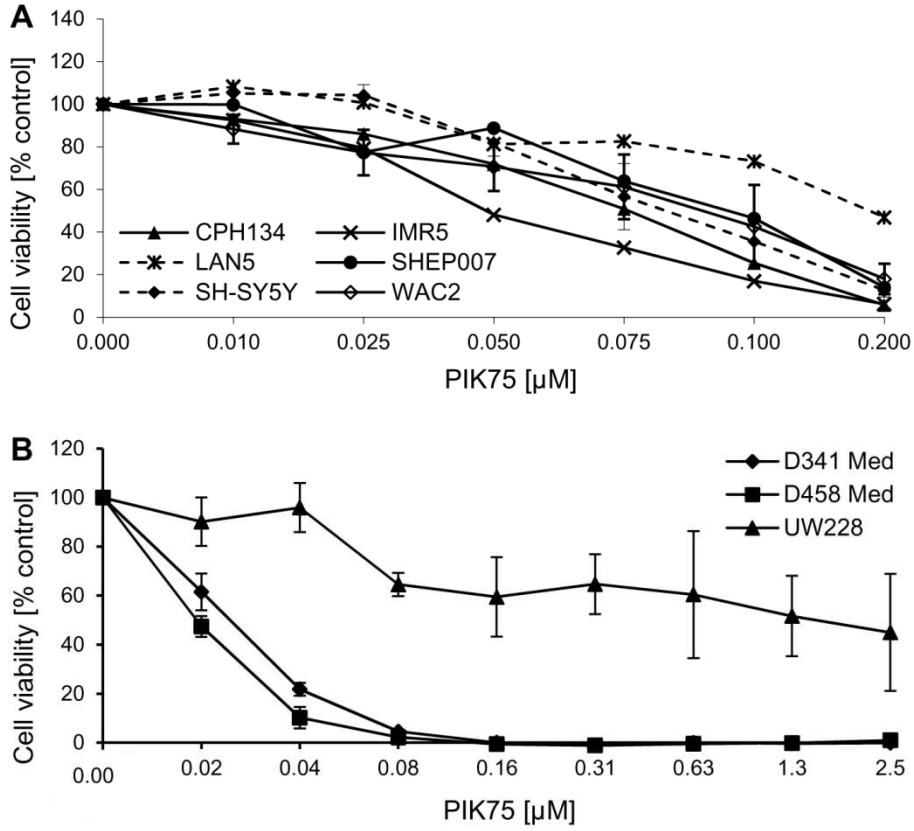


Figure 15. Cell proliferation of NB and MB cells after inhibition of the PI3K p110α. A panel of NB cell lines (A) and MB cell lines (B) were incubated with increasing concentrations of the specific pharmacological PI3K p110α inhibitor PIK75 in serum-containing medium. Cell viability was assessed using the MTS assay after 2 (NB) or 3 (MB) days. The data represent the mean with SD from at least four replicates and 1-3 independent experiment.

Impact of R1507 and PIK75 on intracellular signaling pathway activation

The impact of R1507 and PIK75 on the activation status of the Akt/mTOR pathway in NB cell lines was investigated by Western blot analysis (Fig. 16). R1507 strongly affected the activation status of Akt and the phosphorylation of the mTOR downstream target ribosomal S6 protein in the R1507-responsive NB and MB cell lines. (SH-SY5Y in Fig. 16A, LAN1 cells Fig. 16B, as well as in medulloblastoma PFSK cells Fig. 16D). Concentrations of 6.25-100 µg/ml R1507 reduced Akt^{Ser473} phosphorylation, whereas only concentrations of more than 6.25 or 12.5 were needed to reduce $S6^{Ser235/236}$ phosphorylation. The R1507-insensitive NB cell line WAC2 did not show comparable responses on the PI3K pathway signaling activation upon R1507 treatment (Fig. 16C).

Also PIK75 was able to inhibit Akt/mTOR activation, as seen on the decreases in the phosphorylation of S6 protein in both NB cell lines treated (Fig 16A+B), although only in LAN-1 cells the phosphorylation of Akt^{Ser473} was affected (Fig. 16B). The inhibitory effects of PIK75 in Akt/mTOR signaling in MB cell lines cells were previously described in (284) and correlate with the effects observed in neuroblastoma cells.

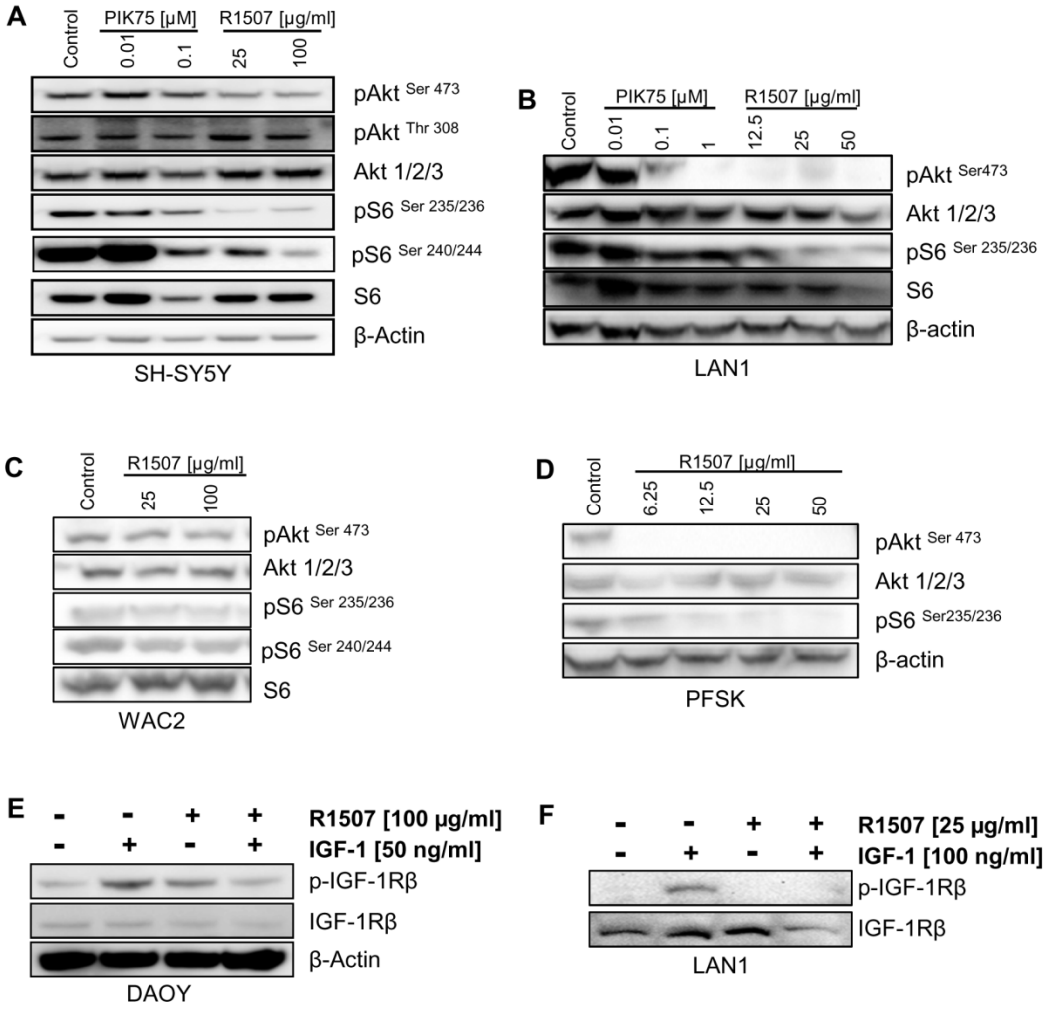


Figure 16. PI3K and IGF-IR inhibition impair receptor activation and downstream signaling. The NB cells SH-SY5Y (A), LAN1 (B); WAC2 (C) and the MB cells PFSK (D) grown in serum-containing medium were incubated with increasing concentrations of the PI3K p110α inhibitor PIK75 and the IGF-IR antibody R1507. After 24 hours the cells were harvested and whole cell lysates analysed by SDS-PAGE and Western blotting for the proteins indicated. (E+F) Serum-starved DAOY (E) and LAN1 (F) cells were pre-treated with vehicle or R1507 at the concentrations indicated for 1h and stimulated with IGF-I (50 or 100 ng/ml) for 10 min at 37°C. Cell lysates were analyzed by SDS-PAGE and Western Blot for phosphorylated IGF-IR beta and total receptor.

IGF-IR expression and activation in neuroblastoma and medulloblastoma cell lines

Further, we wanted to investigate the impact of R1507 and PIK75 on the expression and activation state on the IGF-I receptor in MB and NB cell lines. Pre-treatment with R1507 inhibited the phosphorylation but not the expression level of IGF-IR in DAOY cells after IGF-I stimulation (Fig. 16E). In LAN1 cells the activation state of the IGF-IR was not only decreased by its direct inhibition with R1507 (Fig. 16F) but also by targeting its downstream signaling molecule p110α (data not shown).

Combination of R1507 with standard chemotherapeutic agents in neuroblastoma and medulloblastoma cell lines

We have previously shown that the IGF-IR tyrosine kinase inhibitor NVP-AEW541 enhances the effects of cisplatin on cell proliferation and apoptosis in neuroblastoma cell lines (326). In support of this finding, the concomitant treatment of the R1507-responsive SH-SY5Y neuroblastoma cell line with R1507 and cisplatin resulted in additive effects on cell proliferation (Fig. 17A). For neuroblastoma WAC2 cells that did not respond to R1507 in single treatment (Fig 14A), there was no additional effect of R1507 in combination with cisplatin, doxorubicin or etoposide (Suppl. Fig. 7). In medulloblastoma PFSK and UW228 cells, the combination of R1507 and cisplatin was more effective than the single agents (Fig. 18 A+B). This is not surprising for PFSK, that also showed sensitivity to R1507 alone, but in UW228, cisplatin seems to confer R1507 sensibility. In R1507-insensitive DAOY cells no such effect was observed (Fig. 18C).

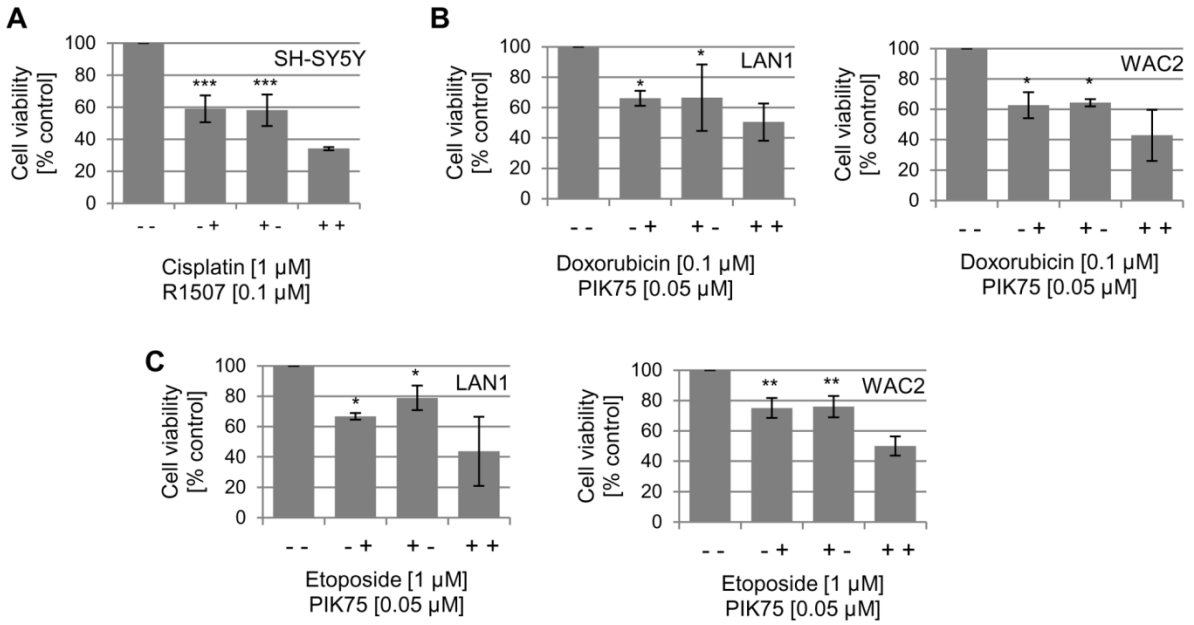


Figure 17. Treatment of NB cells with R1507 or PIK75 in presence of chemotherapy results in additive effects. NB cells grown in serum-containing medium were incubated with the IGF-IR antibody R1507 (0.1 µg/ml) (A) or the PI3K inhibitor PIK75 (0.05 µM) (B+C) in presence or absence of cisplatin (1 µM), etoposide (1 µM), or doxorubicin (0.1 µM). Cell proliferation was assessed using the MTS assay after 48h. The data represent the mean of 8 replicates with SD from3 independent experiments. (*p < 0.05).

Combination of PIK75 with standard chemotherapeutic agents in neuroblastoma cells

In the neuroblastoma cell lines LAN1 and WAC2, the concomitant treatment with PIK75 and doxorubicin or etoposide resulted in additive effects on cell proliferation (Fig. 17B+C). At selected, relatively low concentrations, where PIK75, doxorubicin or etoposide alone reduced cell proliferation to 60-80%, combination treatments of PIK75 and one of the chemotherapeutic agents brought reductions to 40-50%. Our previous work on medulloblastoma has shown that PIK75 sensitizes medulloblastoma cell lines to doxorubicin (284).

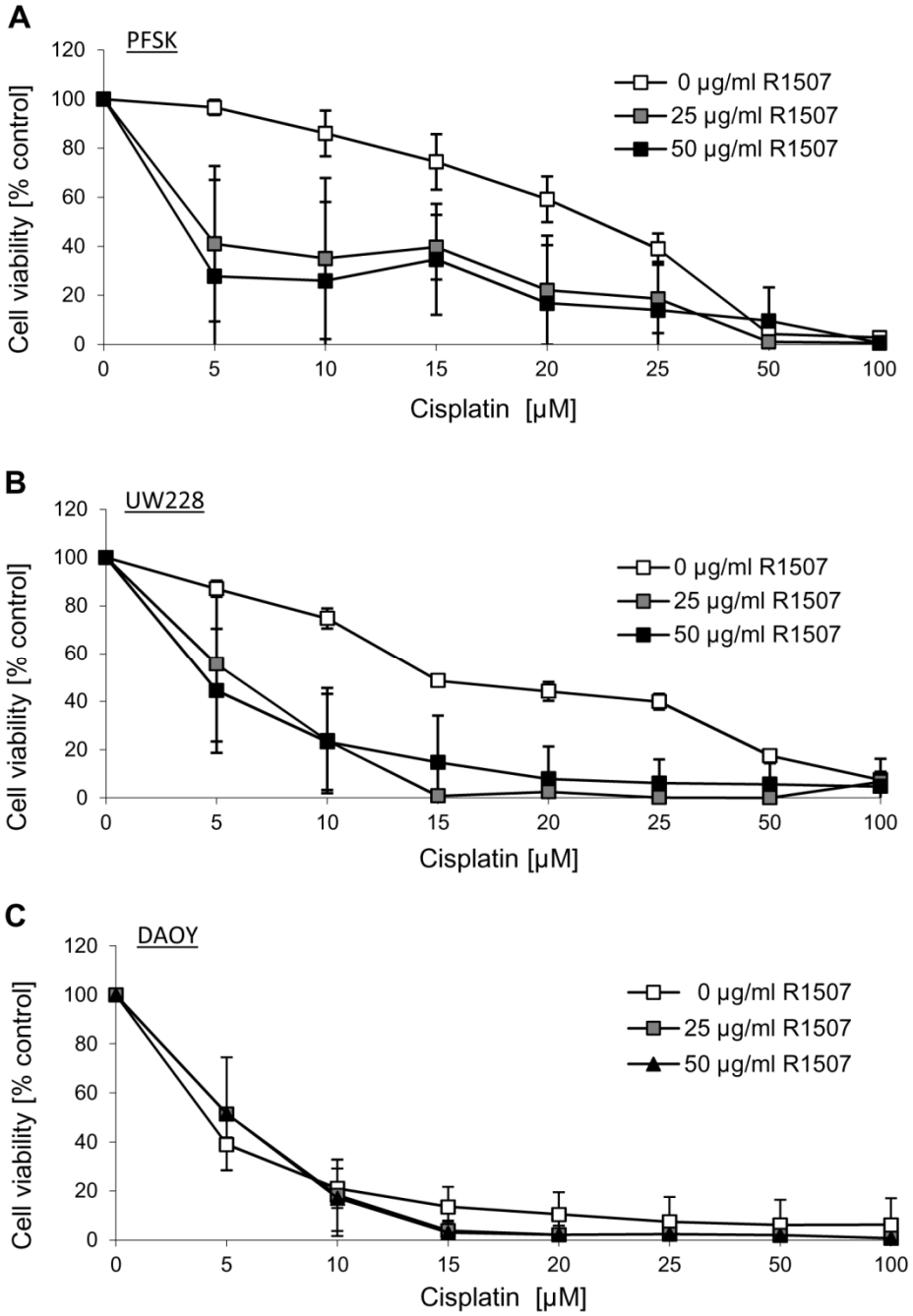


Figure 18. Additive/sensitization effects after combinatorial treatment with R1507 and chemotherapy in MB cells. The MB cell lines PFSK (A), UW228 (B) and DAOY (C) grown in serum-containing medium were incubated with increasing concentrations of cisplatin in presence or absence of the IGF-IR antibody R1507 (25 µg/ml or 50 µg/ml). Cell proliferation was assessed using the MTS assay after 48h. The data represent the mean of 6 replicates with SD from 3 independent experiments

Inhibitors of the RTK-PI3K-mTOR signaling in medulloblastoma

Beside classical chemotherapeutic agents used for cancer treatment, targeted therapies involving inhibitors of the RTK-PI3K signaling axis are considered to be a promising approach in cancer treatment. To investigate the role of receptor tyrosine kinases signaling in medulloblastoma, the effect of different targeted therapies was additionally studied. *In vitro*, the IGF-IR inhibitor NVP-AEW541 reduced the cell viability of the MB cell line DAOY with an IC50 of 2.5 µM and a maximal reduction

of cell proliferation to 5% (Fig. 19A, 20C), whereas in UW228 cells concentrations higher than 10 µM were needed to provoke a response (Fig. 19B). Also targeting the EGFR with gefinitib or erlotinib was more effective in DAOY than in UW228 cells (Fig. 19A+B), with DAOY responding from concentrations higher than 2.5 µM. In UW228 cells erlotinib did not cause any effect, whereas gefitinib treatment reduced cell viability to 5% at the highest concentration tested (20 µM) (Fig. 19B). Rapamycin, a commonly used mTOR inhibitor, led to 50% reduction in cell proliferation in DAOY cells (0.0625 µg/ml) (Fig. 20D). UW228 cells responded to rapamycin with a maximum decrease in cell viability of 30% (2 µg/ml) (Fig. 19B). Imatinib, an inhibitor of the RTKs PDGFR and c-Kit, was able to reduce the cell proliferation in DAOY cells to 15% (Fig. 19A, 20B) and in UW228cells to 40% (Fig. 19B). Before, it could be shown that the concomitant treatment of the IGF-IR antibody R1507 with cisplatin resulted in additive effects in R1507-responsive cell lines, or even was able to sensitize the R1507-non-responsive cell line UW228, but not DAOY (Fig. 18). Furthermore it was of interest, whether a concomitant treatment of different RTK/PI3K/mTOR inhibitors and R1507 could cause sensitization effects in non-responsive DAOY cells. Co-targeting EGFR (gefitinib) or PDGFR and c-Kit (imatinib) together with the IGF-IR (R1507) was not able to sensitize DAOY cells, and interestingly, even caused negative effects, meaning the combination treatment was less effective than the single agent (Fig. 20A+B). R1507, in combination with the IGF-IR inhibitor NVP-AEW541 or the mTOR inhibitor rapamycin could not further increase the effect of the single treatment (Fig. 20C+D).

The role of the IGF-IR/PI3K/Akt signaling axis on cell survival in neuroblastoma and medulloblastoma

The impact of the IGF-IR/PI3K/Akt signaling axis on survival of NB and MB cells was investigated by treating the cells with increasing concentrations of R1507 or PIK75, and apoptosis was measured by PARP cleavage and caspase-3activation, both markers of apoptotic activity. Whereas PIK75 treatment led to enhanced PARP and an increase of cleaved caspase 3 expression in LAN1 or respectively a decrease of the pro-caspase 3 expression in SH-SY5Y cells (Fig. 21A+B) or to an increase in the caspase 3/7 activity in WAC2 NB cells (Fig. 21C), these apoptotic signals could not be observed with R1507 treatment in NB cells or by treating the MB cell line PFSK (Fig. 21A+B) and its impact on apoptotic pathways needs further investigation.

Activity of R1507 and PIK75 in chemoresistant NB cell lines

We next investigated whether R1507 or PIK75 had also anti-proliferative effects in neuroblastoma cell lines with acquired resistance to standard chemotherapeutic agents (LAN1R). R1507 displayed no significant anti-proliferative activity in LAN1 cells with acquired resistance to doxorubicin (Fig. 22A), a result which also could be observed in parental LAN1 cells where silencing reduced the expression level of IGF-IR (Fig. 22D). In contrast, PIK75 displayed almost comparable anti-proliferative activity in either parental LAN1 or their chemoresistant counterparts (Fig. 22B). Western blot analysis of the

protein expression of LAN1R cells showed that these cells express reduced levels of IGF-IR and p110α compared to the parental cell line LAN1. In addition, the phosphorylation levels of ERK1/2 and AKT at the positions Ser^{473} and Thr^{308} were also lower in LAN1R than in LAN1 (FIG 22C).

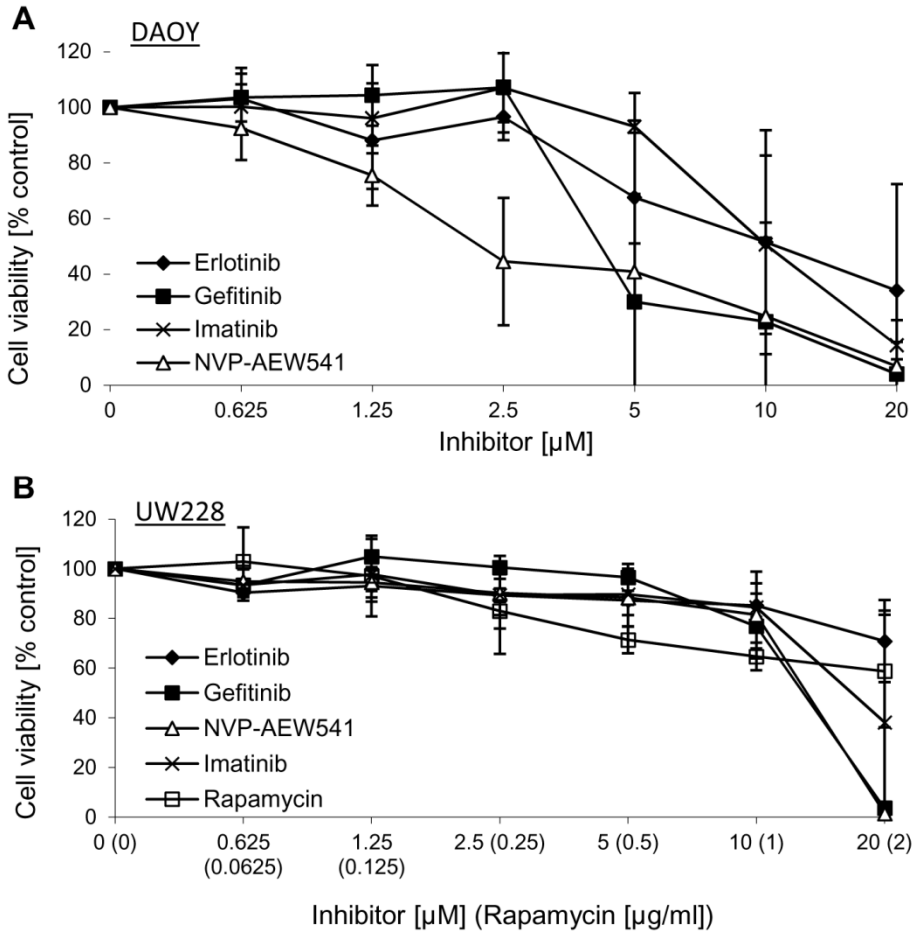


Figure 19. Targeted therapies in medulloblastoma. The MB cell lines Daoy (A) and UW228 (B) were incubated with increasing concentrations of the EGFR inhibitors gefinitib or erlotinib, the Abl inhibitor imatinib, the IGF-IR inhibitor NVP-AEW541 and the mTOR inhibitor rapamycin. Cell proliferation was assessed using the MTS assay after 72h. The data represent the mean of 6 replicates with SD from 3 independent experiments.

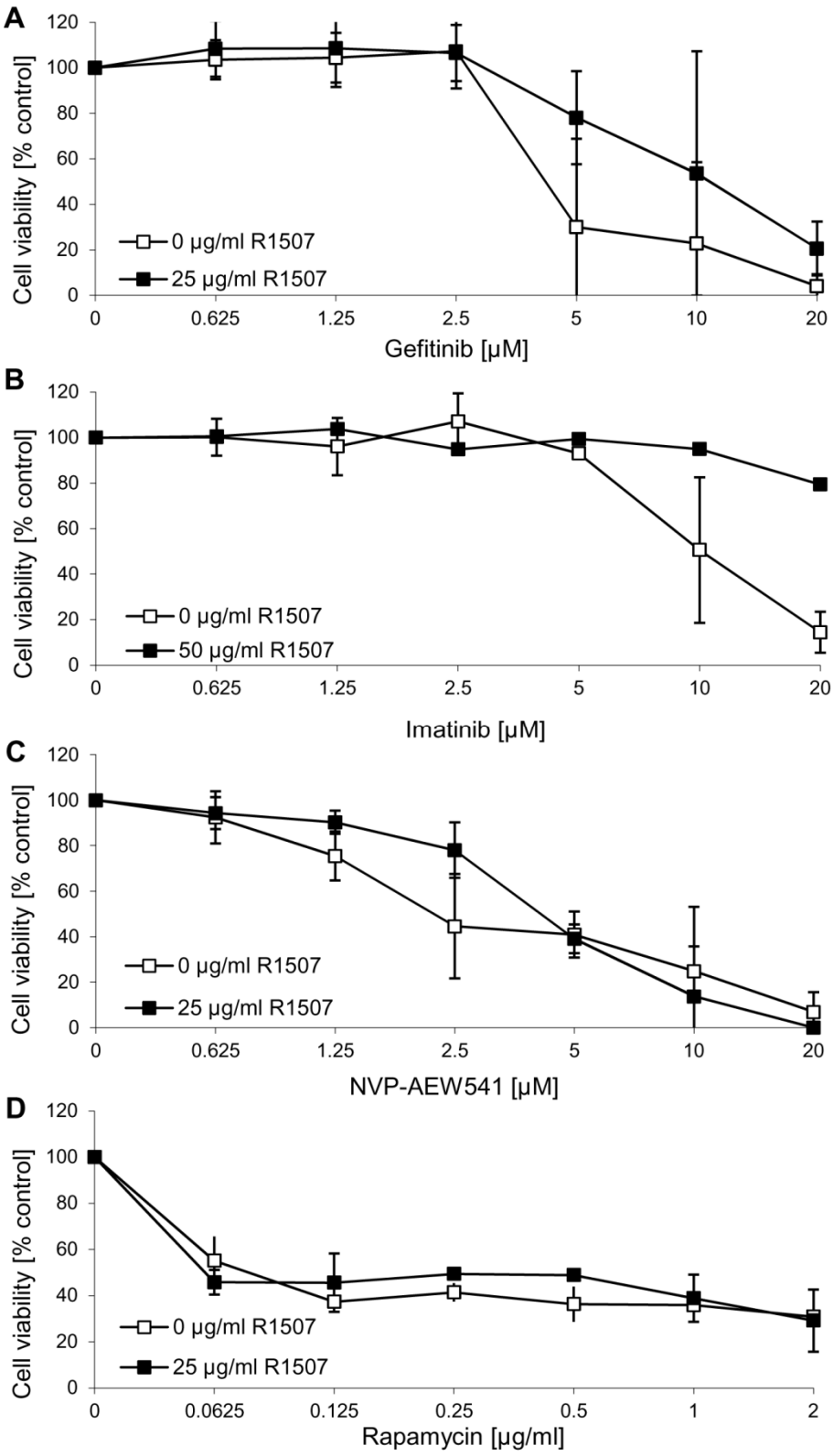


Figure 20. Effects of R1507 in combination with targeted therapies in medulloblastoma. The R1507-insensitive cell line DAOY was incubated with increasing concentrations of the EGFR inhibitor gefinitib (A), the Abl inhibitor imatinib (B), the IGF-IR inhibitor NVP-AEW541 (C) and the mTOR inhibitor rapamycin (D) in presence or absence of the IGF-IR antibody R1507.Cell proliferation was assessed using the MTS assay after 72h. The data represent the mean of 6 replicates with SD from 3 independent experiments.

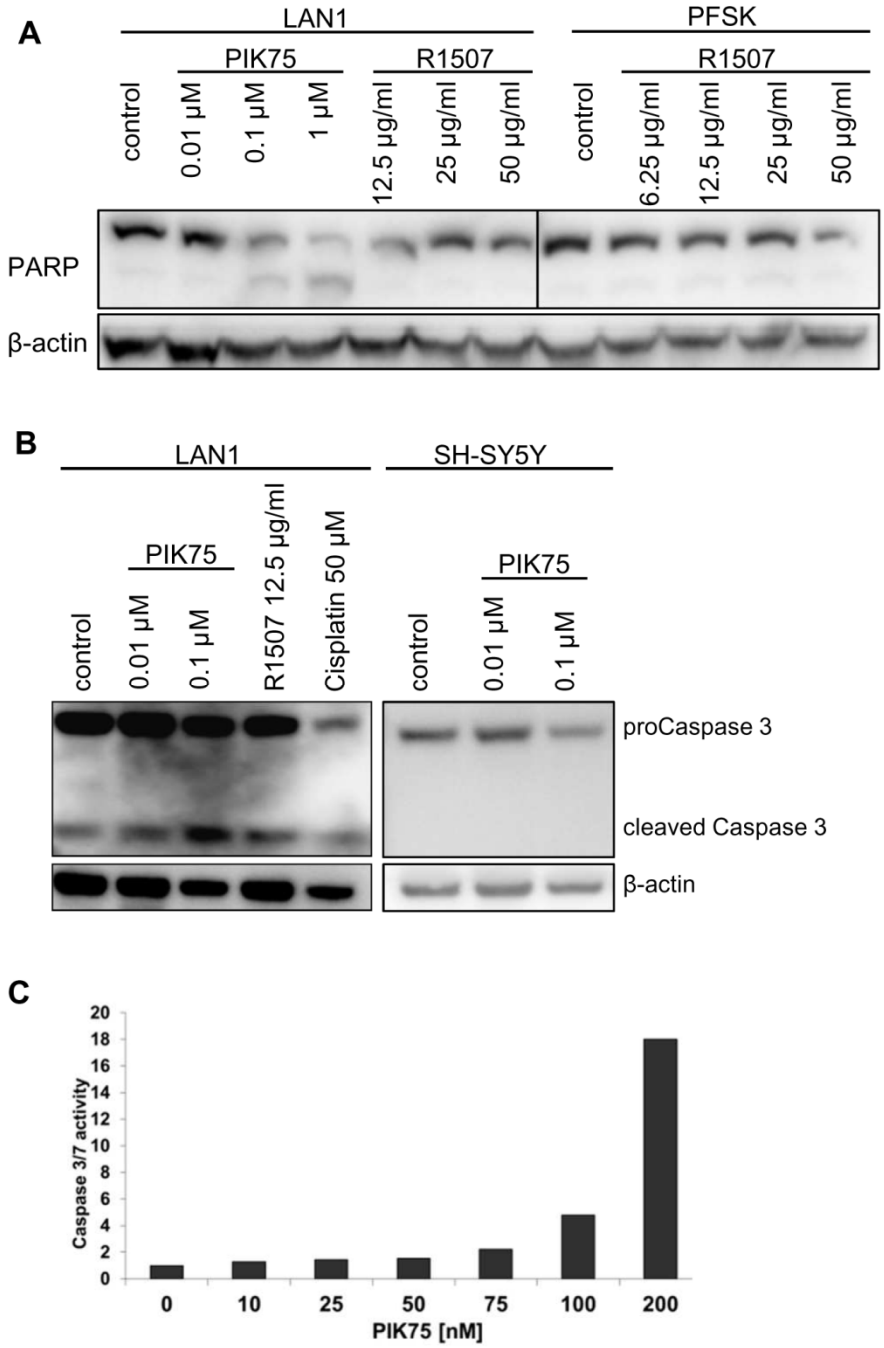


Figure 21. Apoptosis upon PI3K inhibition. (A+B) The NB cell lines LAN1 (A, left panel; B, left panel) and SH-SY5Y (B, right panel) as well as the MB cell line PFSK (A, right panel) grown in serum-containing medium were incubated with increasing concentrations of the PI3K p110α inhibitor PIK75 and the IGF-IR antibody R1507, or cisplatin. After 24 hours the cells were harvested and whole cell lysates analysed by SDS-PAGE and Western blotting for the proteins indicated**.** (C) The NB cell line WAC2 grown in serum-containing medium was incubated with increasing concentrations of the PI3K p110α inhibitor PIK75. Caspase 3/7 activity was assessed using the Caspase 3/7 Glo assay after 48h.

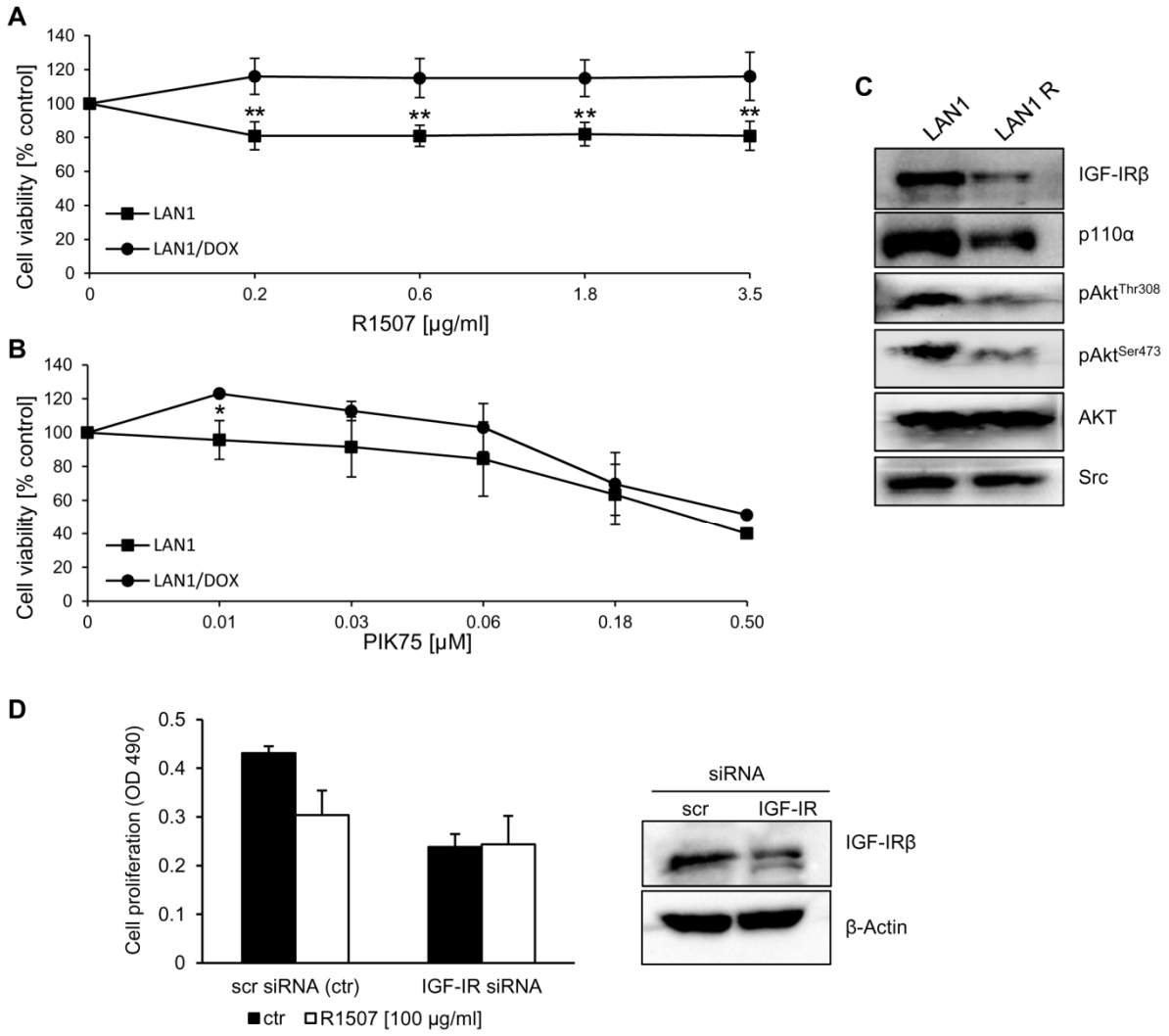


Figure 22. Sensitivity to R1507 and PIK75, and presence of IGFR in neuroblastoma cell lines LAN1 and LAN1R, a LAN1 cell line resistant to doxorubicin. A) R1507 treatment for 48 hours. B) PIK75 treatment for 48 hours. Error bars represent ±S.D. of means from 3 experiments, each with 3 replicates, except that there was only one experiment with 500 nM PIK75 in B. C) Western blot analysis of components of the IGF-IR/PI3K pathway in LAN1 and LAN1R whole cell extracts. Src was used as internal loading control. D) Treatment of LAN1 cells with siRNA targeting the IGF-IR. (A non-targeting construct was used as control). Expression level of IGF-IR was assessed via Western blot analysis in LAN1 whole cell lysates after 96h. Cell proliferation in LAN1 cells upon IGF-IR silencing was assessed in absence or presence of R1507 after 96h by MTS. (*$p < 0.05$).

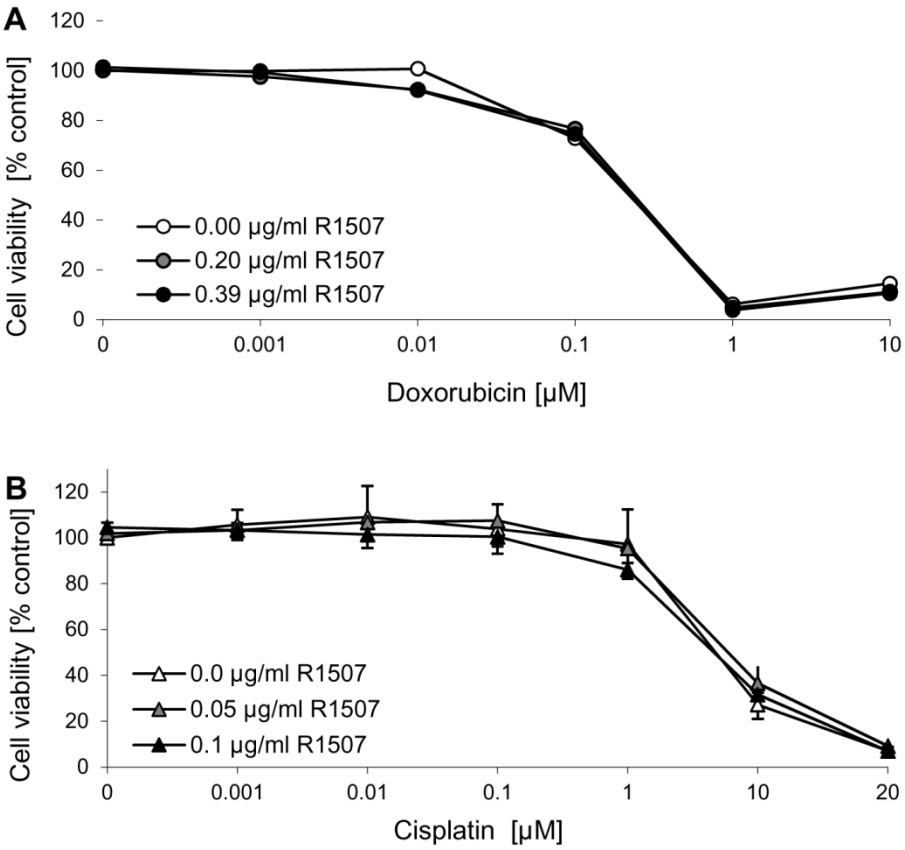
Discussion

In the present report we have evaluated the anti-proliferative activity of the humanized anti-IGF-1R antibody R1507 in the embryonal tumors neuroblastoma and medulloblastoma *in vitro*. As a single agent, R1507 was effective in a subset of neuro- and medulloblastoma cell lines, while a majority of cell lines did not respond. The profile of R1507 in neuro- and medulloblastoma was similar to the IGF-1R tyrosine kinase inhibitor NVP-AEW541 in terms of the identity of the cell lines which were sensitive to the single agent (326). In neuroblastoma cell lines that were sensitive to R1507 as single agent, the effects of R1507 and chemotherapy (cisplatin, doxorubicin and etoposide) were additive, a result which was also observed with NVP-AEW541 (326). However, neuroblastoma cells which were not sensitive to R1507, showed also no additive effects in cell growth inhibition to when combined with chemotherapies. By contrast, in medulloblastoma R1507 showed strong additive effects with cisplatin not only in MB cells which were initially sensitive to R1570 (PFSK), but also MB cells which were insensitive to R1507 as a single agent (UW228). Analysis of the mechanisms of action revealed that R1507 inhibits cell growth by attenuation of the AKT/mTOR signaling pathway in neuroblastoma and medulloblastoma cells. Similar observations were obtained by inhibition of IGF-1R with NVP-AEW541 [20]. Interestingly, the concomitant treatment with R1507 and inhibitors of the RTK/PI3K/mTOR signaling could not overcome the resistance of insensitive DAOY cells, resulted in combination with gefitinib and imatinib even in negative effects, compared to when they were used as single agent. Generally, DAOY cells responded to single treatments targeting the RTK/PI3K/mTOR signaling, such as IGF-IR (NVP-AEW541), EGFR (gefinitib, erlotinib), PDGFR and c-Kit (imatinib), and mTOR (rapamycin). Cell growth of the cell line UW228 was mostly not affected or higher concentrations were needed to induce a response by use of the same agents.

Our previous work using RNAi targeting of classI_A PI3K isoforms has revealed that targeting these enzymes in neuroblastoma and medulloblastoma cell lines can induce apoptosis and decrease cell proliferation (277, 284). These results are supported by the findings presented here, which show that PIK75 displays a broad anti-proliferative activity in neuroblastoma cell lines. Also in medulloblastoma, we observed that PIK75 has anti-proliferative activity, but one cell line (UW228) was rather resistant to the drug. The exact mechanism(s) underlying this observation are at present unclear, but may be caused by an enhanced activation of Erk1/2. A decrease in activity of class I_A PI3K inhibitors has been observed previously in cell lines with mutant *KRAS* and attributed to the enhanced activation of the Erk pathway (368). The combination of PIK75 with chemotherapy (doxorubicin and etoposide) showed enhanced cell growth inhibition as compared with single agent treatment in neuroblastoma cell lines. Consistent with these findings, a recent report demonstrated that PI103 a dual inhibitor against p110α and mTOR strongly synergizes with various chemotherapeutics including doxorubicin, etoposide and cisplatin (369). In medulloblastoma, our previous work has also demonstrated the anti-proliferative effects for PIK75 in combination with different chemotherapeutic agents (284).

Because neuroblastoma and medulloblastoma cells may express a variety of different growth factor receptors, we and others have postulated that targeting individual receptors may not always provide the best therapeutic option (277, 284, 326). To overcome this problem, an alternative approach was proposed, which is based on targeting downstream signaling molecules that are regulated by different growth factor receptors to transmit the proliferative message. Our findings support this approach, since we observed that generally a bigger number of NB and MB cell lines most likely responded to PIK75 than to R1507. Importantly, PIK75 effectively inhibited proliferation in a chemoresistant neuroblastoma cell line in a comparable manner as in the parental cell line, demonstrating its broad anti-proliferative activity. By contrast, R1507 was ineffective in the chemoresistant neuroblastoma cells, which was most likely caused by reduced expression of the IGF-1R. The activation status of the AKT/mTOR pathway was also found to be reduced in the chemoresistant cells, pointing that this signaling pathway may not be responsible for the acquired chemoresistant phenotype of the cells. However, our previous findings in medulloblastoma cells showed elevated levels of phosphorylated Akt as a consequence of short time exposure with doxorubicin (284). The molecular mechanisms underlying these observations are at present unclear, but may be of importance, in view of the fact that some clinical trials have been initiated with R1507 in patients previously treated with chemotherapy (370).

Supplementary Data



Supplementary Figure 7. Neuroblastoma WAC2 cells treated with R1507 in combination with chemotherapy, incubated for 48 hours. Already shown to be insensitive to R1507 alone (Fig. 1A), WAC2 cells are here shown also to be insensitive to R1507 in combination with chemotherapeutic agents A) doxorubicin and B) cisplatin. Error bars represent ±S.D. of means from 1 to 2 experiments, each with 8 replicates. For combination experiments with R1507-sensitive neuroblastoma cell lines, see Figure 4.

5 CONCLUSION & FUTURE PERSPECTIVE

5.1 TARGETING PI3K SIGNALLING IN SMALL CELL LUNG CANCER

In the present study, we demonstrated the class I*A* PI3K isoform p110α to be a critical mediator in controlling SCLC tumour growth, proliferation, and survival processes.

The PI3K/Akt/mTOR pathway has been demonstrated to play a key role in SCLC cell proliferation, survival, chemoresistance and migration. Mutations in *PIK3CA* and gene amplification were reported in primary SCLC, as well as increased expression of *PIK3CA* at the mRNA and protein level. Broad specificity PI3K/mTOR inhibitors have shown anti-tumour activity in SCLC models *in vitro* and *in vivo* (118).

Thus, we sought to investigate the potential of isoform-specific inhibitors and RNAi targeting class I_A PI3K isoforms on SCLC cell responses.

We found, that cell viability and the activation status of classical PI3K downstream targets were highly impaired by agents and RNAi approaches targeting p110α, compared to those targeting p110β or p110δ, which had less or no effect, suggesting a crucial role for p110α in SCLC.

Furthermore, p110α silencing resulted in G1 cell cycle arrest, affected expression of proteins controlling cell cycle progression and to sensitization of commonly used chemotherapeutic agents, such as etoposide and carboplatin.

The important role of p110α and the impact of its inhibition on SCLC survival and tumour growth found in our *in vitro* studies were additionally confirmed in an *in vivo* model. Previously, a significant correlation of VEGF expression and vessel density has been demonstrated in SCLC xenografts and high levels of VEGF were reported to correlate with tumour stage, disease progression, and resistance to chemotherapy, thus leading to a poorer outcome in SCLC patients. We found that angiogenesis *in vivo* and expression of VEGFA *in vitro* were decreased upon p110α inhibition, suggesting p110α signalling to be involved in SCLC angiogenesis.

High expression levels of p110α in the SCLC cell lines under study had been demonstrated before, suggesting its involvement in SCLC biology. Indeed, we also found overexpression of both p110α and p110β in SCLC patient tissue samples, when compared to normal lung tissue. Interestingly, advanced-staged SCLC was accompanied by increased overexpression of p110α, further pointing out its selective role in SCLC.

In view of these observations, we hypothesized that p110α may control the expression of a selective subset of genes implicated in SCLC cell proliferation and/or survival. The comparative DNA microarray analysis of SCLC cell lines in which either p110α or p110β were targeted by selective inhibitors identified such a gene subset.

Among the widespread affected biological categories we identified and validated the Bcl2 family of proteins as a downstream target of p110α, which displayed an imbalance in the expression of anti-apoptotic and pro-apoptotic Bcl2 proteins upon p110α silencing. The Bcl2 family of proteins has been

previously shown to play a crucial role in the survival of SCLC cell lines *in vitro* and *in vivo* (227). Bcl2 inhibition was already shown to suppress SCLC growth and survival which is consistent with the results presented in this study.

In view of the overexpression of p110α in SCLC tumours, the observation that Bcl2 expression was elevated in primary SCLC further supports the model of Bcl2 as a downstream target of p110α in SCLC. Bcl2 is a key regulator of both apoptosis and autophagy (306) and its transcription is known to be regulated by the transcription factor NFκB. Indeed, we did not only find induction of SCLC cell death via apoptosis and autophagy by the p110α inhibitors, but also a deregulated transcriptional network of NFκB. Thus, revealing the transcription factor NFκB being a downstream target of p110α and suggesting an essential role of the p110α/NFκB signalling axis mediating pro-survival signals in SCLC.

Interestingly, *PIK3CA* transcription has been already demonstrated to be positively controlled by the NFκB (371), thus, giving rise to the speculation of the existence of feedback loops and their regulation in SCLC. In contrast, the tumour suppressor p53 acts as negative regulator on *PIK3CA* transcription. The majority of SCLC tumours display mutations in the *TP53* gene (90%) and dys-functionality of its protein product p53 (40-70%), which could lead to the assumption of a correlation between the activation and overexpression of p110α and the dysfunctional expression of p53 in SCLC.

We have previously evaluated the mTOR inhibitor everolimus in SCLC cell lines and found that it was effective in a subset of SCLC cell lines characterized by activation of the Akt/mTOR pathway and low expression levels of anti-apoptotic Bcl2 family proteins (227). In view of the present results obtained with isoform-selective inhibitors of p110α, it can be speculated that these agents may be more potent, since they induce a down-regulation of Bcl2 and of the activity of the Akt/mTOR pathway.

Our previous work has shown that the class I_A PI3K isoform p110δ contributes to cell proliferation and survival in neuroblastoma by controlling the mTOR pathway activation and the expression levels of anti-apoptotic Bcl2 family proteins. Over-expression of p110δ was found in a subset of primary neuroblastoma and cell lines, and p110δ was essential for signal transduction by receptor tyrosine kinases, such as the IGF-IR and EGFR (277). In contrast, in SCLC cell lines, p110α appears to play a more particular role for mediating RTK signalling, which may explain the impact of agents targeting this isoform on SCLC proliferation and survival. A previous report has shown that the activity of any class I_A PI3K isoform can maintain cell survival (227). Therefore, the relative importance of class I_A PI3K isoforms in selected cancer types may be, in part, attributed to differences in expression levels.

Taken together, targeting PI3K p110α signalling may represent an attractive novel approach to develop novel therapies for SCLC. Indeed, there now exist several pharmacological inhibitors of this isoform, which will soon enter clinical trials in oncology, and could, in the future, be developed as new drugs for SCLC.

5.2 Targeting the IGF-IR/PI3K Signalling Axis in Embryonal Tumours

In the present study, we demonstrated that targeting the class I PI3K isoform p110α in embryonal tumours clearly displays advantage as anti-cancer approach over the inhibition of the IGF-IR *in vitro*.

We have evaluated the anti-proliferative activity of the humanized anti-IGF-IR antibody R1507 in the embryonal tumours neuroblastoma and medulloblastoma *in vitro*. By targeting the IGF-IR/PI3K signalling axis we observed cell line-specific responses and for some cell lines a strong decrease in cell proliferation.

As a single agent, R1507 was effective in a subset of neuro- and medulloblastoma cell lines, while a majority of cell lines did not respond. The response profile of R1507 in neuro- and medulloblastoma was similar to the IGF-IR tyrosine kinase inhibitor in terms of the identity of the cell lines which were sensitive to the single agent. Additionally, the mechanisms of action revealed that R1507 inhibits cell growth by attenuation of the AKT/mTOR signalling pathway in neuroblastoma and medulloblastoma cells, as it was the case for NVP-AEW541 (372) Interestingly, NB cells which were sensitive to single agent R1507 could be sensitized to chemotherapeutic agents, which was also observed with NVP-AEW541 (372). However, R1507-resistant neuroblastoma cells, showed also no additive effects in cell growth inhibition to when combined with chemotherapies.

By contrast, concomitant treatment with R1507 and cisplatin resulted in strong additive effects not only in sensitive but also in MB cells which were initially resistant to R1570 as single agent.

Surprisingly, the concomitant treatment with R1507 and inhibitors of the RTK/PI3K/mTOR signalling could not overcome the resistance of insensitive DAOY MB cells, caused even negative effects in combination with gefitinib and imatinib, even though positive responses could be observed by the use of single agent RTK inhibitors.

Our previous work using RNAi targeting of class I_A PI3K isoforms has revealed that targeting these enzymes in neuroblastoma and medulloblastoma cell lines can induce apoptosis and decrease cell proliferation (227). These results are consistent with the findings presented here, which show that p110α inhibition displays a broad anti-proliferative activity in NB and MB cell lines, with one single MB one cell line being rather resistant to the drug. The exact mechanism(s) underlying this observation still remain unclear, but may be caused by an enhanced activation of the MAPK/Erk1/2 signalling. A decrease in activity of class I_A PI3K inhibitors has been observed previously in cell lines with mutant *KRAS* and attributed to the enhanced activation of the Erk pathway (227).

In MB, our previous work has demonstrated the anti-proliferative effects for p110α inhibition in combination with different chemotherapeutic agents (227). Here, these results could be confirmed in NB. The combination of PIK75 with chemotherapy resulted in enhanced cell growth suppression as compared with single agent treatment. Consistent with these findings, a recent report demonstrated that PI103 a dual inhibitor against p110α and mTOR strongly synergizes with various chemotherapeutics including doxorubicin, etoposide and cisplatin (227).

We observed, that generally a larger number of NB and MB cell lines responded to PIK75 than to R1507. This might be due to the activation of PI3K downstream of numerous RTKs expressed in NB and MB, suggesting that targeting one single RTK may not be the best treatment approach (227). Additionally, p110α inhibition caused anti-proliferative effects in a chemoresistant NB cell line, whereas R1507 was ineffective, which was most likely caused by the reduced expression of the IGF-IR. The activation status of the AKT/mTOR pathway was also found to be reduced in the chemoresistant cells, pointing that this signalling pathway may not be responsible for the acquired chemoresistant phenotype of the cells. However, our previous findings in medulloblastoma cells showed elevated levels of phosphorylated Akt as a consequence of short time exposure with doxorubicin (227). The molecular mechanisms underlying these observations are at present unclear, but may be of importance, in view of the fact that some clinical trials have been initiated with R1507 in patients previously treated with chemotherapy (370).

5.3 Concluding Remarks

The importance of the PI3K signalling pathway, which controls cell survival and proliferation processes, and its impact on cancer development have been demonstrated in the last two decades in various human neoplasms. The escape of atypical cells from the normal growth control, in addition to deregulation of PI3K signalling and closely related pathways drives cells into a malignant and invasive phenotype. Deregulation of these pathways results in a lack of response to negative growth regulatory signals and the continuous presence of positive signals involving the RTK/PI3K/Akt pro-survival axis. Therefore, targeting RTK/PI3K/Akt signalling with small molecule inhibitors has become an interesting and promising approach in cancer therapy development. Indeed, there are now several targeted agents directed against the PI3K/Akt/mTOR pathway which have entered clinical trials in numerous cancer types. The use of targeted therapies in addition to commonly administered chemotherapy could improve clinical benefit for patients, especially in patients with cancer types depending on the presence of one particular oncogene. Because cancer cells may express a variety of different growth factor receptors it was postulated that targeting individual receptors may not always provide the best therapeutic option. To overcome this problem, an alternative approach was proposed, which is based on targeting downstream signalling molecules that are regulated by multiple growth factor receptors to transmit their proliferative message. The findings presented here and in numerous studies support this model. Additionally, even though the whole “cancer cell machinery” is not yet adequately understood and there exist many “missing links”, the growing knowledge about genetic alterations driving cancer development and maintenance provides the possibility to identify predictive molecular markers, which are of importance for further therapy development and future translation to clinical trials.

6 LIST OF ABBREVIATIONS

4EBP1	eukaryotic translation initiation factor 4E binding protein 1
Akt	v-akt murine thymoma viral oncogene homolog 1
AT/RT	atypical teratoid/rhabdoid tumors
ATP	adenosine triphosphate
Bad	Bcl2-associated death promoter
Bax	Bcl2-associated X protein
Bcl2	B-cell leukemia/lymphoma 2
$BclX_L$	B-cell leukemia/lymphoma extra large
BIRC5	survivin
cDNA	complementary DNA
c-Met	mesenchymal epithelial transition factor receptor (hepatocyte growth factor receptor)
CNS	central nervous system
EGFR	epidermal growth factor receptor
ErbB2	erythroblastic leukemia viral oncogene homolog 2
Erk1/2	extracellular signal-regulated kinase 1/2
GPCR	G protein-coupled receptor
GSK	glycogen synthase kinase
HGF	hepatocyte growth factor
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding protein
IGFIR	insulin-like growth factor I receptor
INSS	International Neuroblastoma Staging System
IR	insulin receptor
IRS	insulin receptor substrate
LOH	loss of heterozygosity
MAPK	mitogen-activated protein kinase
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
mTORC1	mammalian target of rapamycin complex 1
mTORC2	mammalian target of rapamycin complex 2
MB	medulloblastoma
NB	neuroblastoma
NSCLC	Non-small cell lung cancer
PDGF	platelet-derived growth factor
PDK1	phosphoinositide-dependent kinase 1
PIK3C	genes encoding for the PI3K
PI	phosphatidylinositol
PI3K	phosphatidylinositol 3-kinase
PI(3)P	phosphatidylinositol 3-phosphate
PI(3,4)P2	phosphatidylinositol (3,4)-bisphosphate
PI(3,4,5)P3	phosphatidylinositol (3,4,5)-trisphosphate
PI3K	Phosphatidylinositol 3-kinase/phophoinositide 3-kinase
PTEN	phosphatase and tensin homolog
qRT-PCR	quantitative real-time PCR
Rheb	Ras homologue enriched in the brain
RNAi	RNA interference
RT	radiotherapy
RTK	receptor tyrosine kinase
S6K	ribosomal protein S6 kinase
SCF	stem cell factor
SCLC	small cell lung cancer
SH2	Src homology-2
Shh	sonic hedgehog
shRNA	small-hairpin RNA
siRNA	small-interfering RNA
TSC1	TSC2 tuberous sclerosis complex 1, 2
WHO	World Health Organization
Wnt	wingless

7 REFERENCES

1. Katso, R., Okkenhaug, K., Ahmadi, K., White, S., Timms, J., and Waterfield, M. D. Cellular function of phosphoinositide 3-kinases: implications for development, homeostasis, and cancer. Annu Rev Cell Dev Biol, *17:* 615-675, 2001.
2. Cantley, L. C. The phosphoinositide 3-kinase pathway. Science, *296:* 1655-1657, 2002.
3. Lemmon, M. A. Pleckstrin homology (PH) domains and phosphoinositides. Biochem Soc Symp 81-93, 2007.
4. Xu, Y., Seet, L. F., Hanson, B., and Hong, W. The Phox homology (PX) domain, a new player in phosphoinositide signalling. Biochem J, *360:* 513-530, 2001.
5. Engelman, J. A. Targeting PI3K signalling in cancer: opportunities, challenges and limitations. Nat Rev Cancer, *9:* 550-562, 2009.
6. Robinson, D. R., Wu, Y. M., and Lin, S. F. The protein tyrosine kinase family of the human genome. Oncogene, *19:* 5548-5557, 2000.
7. Hubbard, S. R., Mohammadi, M., and Schlessinger, J. Autoregulatory mechanisms in protein-tyrosine kinases. J Biol Chem, *273:* 11987-11990, 1998.
8. Jiang, G. and Hunter, T. Receptor signaling: when dimerization is not enough. Curr Biol, *9:* R568-571, 1999.
9. Schlessinger, J. Cell signaling by receptor tyrosine kinases. Cell, *103:* 211-225, 2000.
10. Engelman, J. A., Chen, L., Tan, X., Crosby, K., Guimaraes, A. R., Upadhyay, R., Maira, M., McNamara, K., Perera, S. A., Song, Y., Chirieac, L. R., Kaur, R., Lightbown, A., Simendinger, J., Li, T., Padera, R. F., Garcia-Echeverria, C., Weissleder, R., Mahmood, U., Cantley, L. C., and Wong, K. K. Effective use of PI3K and MEK inhibitors to treat mutant Kras G12D and PIK3CA H1047R murine lung cancers. Nat Med, *14:* 1351-1356, 2008.
11. Liu, P., Cheng, H., Roberts, T. M., and Zhao, J. J. Targeting the phosphoinositide 3-kinase pathway in cancer. Nat Rev Drug Discov, *8:* 627-644, 2009.
12. Engelman, J. A., Luo, J., and Cantley, L. C. The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. Nat Rev Genet, *7:* 606-619, 2006.
13. Antonetti, D. A., Algenstaedt, P., and Kahn, C. R. Insulin receptor substrate 1 binds two novel splice variants of the regulatory subunit of phosphatidylinositol 3-kinase in muscle and brain. Mol Cell Biol, *16:* 2195-2203, 1996.
14. Fruman, D. A., Cantley, L. C., and Carpenter, C. L. Structural organization and alternative splicing of the murine phosphoinositide 3-kinase p85 alpha gene. Genomics, *37:* 113-121, 1996.
15. Inukai, K., Anai, M., Van Breda, E., Hosaka, T., Katagiri, H., Funaki, M., Fukushima, Y., Ogihara, T., Yazaki, Y., Kikuchi, Oka, Y., and Asano, T. A novel 55-kDa regulatory subunit for phosphatidylinositol 3-kinase structurally similar to p55PIK Is generated by alternative splicing of the p85alpha gene. J Biol Chem, *271:* 5317-5320, 1996.
16. Inukai, K., Funaki, M., Ogihara, T., Katagiri, H., Kanda, A., Anai, M., Fukushima, Y., Hosaka, T., Suzuki, M., Shin, B. C., Takata, K., Yazaki, Y., Kikuchi, M., Oka, Y., and Asano, T. p85alpha gene generates three isoforms of regulatory subunit for phosphatidylinositol 3-kinase (PI 3-Kinase), p50alpha, p55alpha, and p85alpha, with different PI 3-kinase activity elevating responses to insulin. J Biol Chem, *272:* 7873-7882, 1997.
17. Krugmann, S., Hawkins, P. T., Pryer, N., and Braselmann, S. Characterizing the interactions between the two subunits of the p101/p110gamma phosphoinositide 3-kinase and their role in the activation of this enzyme by G beta gamma subunits. J Biol Chem, *274:* 17152-17158, 1999.
18. Suire, S., Hawkins, P., and Stephens, L. Activation of phosphoinositide 3-kinase gamma by Ras. Curr Biol, *12:* 1068-1075, 2002.
19. Hirsch, E., Katanaev, V. L., Garlanda, C., Azzolino, O., Pirola, L., Silengo, L., Sozzani, S., Mantovani, A., Altruda, F., and Wymann, M. P. Central role for G protein-coupled phosphoinositide 3-kinase gamma in inflammation. Science, *287:* 1049-1053, 2000.
20. Vanhaesebroeck, B., Leevers, S. J., Panayotou, G., and Waterfield, M. D. Phosphoinositide 3-kinases: a conserved family of signal transducers. Trends Biochem Sci, *22:* 267-272, 1997.
21. Vanhaesebroeck, B., Welham, M. J., Kotani, K., Stein, R., Warne, P. H., Zvelebil, M. J., Higashi, K., Volinia, S., Downward, J., and Waterfield, M. D. P110delta, a novel phosphoinositide 3-kinase in leukocytes. Proc Natl Acad Sci U S A, *94:* 4330-4335, 1997.
22. Falasca, M. and Maffucci, T. Regulation and cellular functions of class II phosphoinositide 3-kinases. Biochem J, *443:* 587-601, 2012.
23. Schu, P. V., Takegawa, K., Fry, M. J., Stack, J. H., Waterfield, M. D., and Emr, S. D. Phosphatidylinositol 3-kinase encoded by yeast VPS34 gene essential for protein sorting. Science, *260:* 88-91, 1993.
24. Wurmser, A. E., Gary, J. D., and Emr, S. D. Phosphoinositide 3-kinases and their FYVE domain-containing effectors as regulators of vacuolar/lysosomal membrane trafficking pathways. J Biol Chem, *274:* 9129-9132, 1999.

25. Gulati, P., Gaspers, L. D., Dann, S. G., Joaquin, M., Nobukuni, T., Natt, F., Kozma, S. C., Thomas, A. P., and Thomas, G. Amino acids activate mTOR complex 1 via Ca2+/CaM signaling to hVps34. Cell Metab, *7:* 456-465, 2008.
26. Nobukuni, T., Joaquin, M., Roccio, M., Dann, S. G., Kim, S. Y., Gulati, P., Byfield, M. P., Backer, J. M., Natt, F., Bos, J. L., Zwartkruis, F. J., and Thomas, G. Amino acids mediate mTOR/raptor signaling through activation of class 3 phosphatidylinositol 3OH-kinase. Proc Natl Acad Sci U S A, *102:* 14238-14243, 2005.
27. Backer, J. M. The regulation and function of Class III PI3Ks: novel roles for Vps34. Biochem J, *410:* 1-17, 2008.
28. Marone, R., Cmiljanovic, V., Giese, B., and Wymann, M. P. Targeting phosphoinositide 3-kinase: moving towards therapy. Biochim Biophys Acta, *1784:* 159-185, 2008.
29. Manning, B. D. and Cantley, L. C. AKT/PKB signaling: navigating downstream. Cell, *129:* 1261-1274, 2007.
30. Chalhoub, N. and Baker, S. J. PTEN and the PI3-kinase pathway in cancer. Annu Rev Pathol, *4:* 127-150, 2009.
31. Hengartner, M. O. The biochemistry of apoptosis. Nature, *407:* 770-776, 2000.
32. Hanahan, D. and Weinberg, R. A. Hallmarks of cancer: the next generation. Cell, *144:* 646-674, 2011.
33. Hotchkiss, R. S., Strasser, A., McDunn, J. E., and Swanson, P. E. Cell death. N Engl J Med, *361:* 1570-1583, 2009.
34. Falschlehner, C., Emmerich, C. H., Gerlach, B., and Walczak, H. TRAIL signalling: decisions between life and death. Int J Biochem Cell Biol, *39:* 1462-1475, 2007.
35. Schutze, S., Tchikov, V., and Schneider-Brachert, W. Regulation of TNFR1 and CD95 signalling by receptor compartmentalization. Nat Rev Mol Cell Biol, *9:* 655-662, 2008.
36. Rothe, M., Pan, M. G., Henzel, W. J., Ayres, T. M., and Goeddel, D. V. The TNFR2-TRAF signaling complex contains two novel proteins related to baculoviral inhibitor of apoptosis proteins. Cell, *83:* 1243-1252, 1995.
37. Szegezdi, E., Macdonald, D. C., Ni Chonghaile, T., Gupta, S., and Samali, A. Bcl-2 family on guard at the ER. Am J Physiol Cell Physiol, *296:* C941-953, 2009.
38. Hoetelmans, R., van Slooten, H. J., Keijzer, R., Erkeland, S., van de Velde, C. J., and Dierendonck, J. H. Bcl-2 and Bax proteins are present in interphase nuclei of mammalian cells. Cell Death Differ, *7:* 384-392, 2000.
39. Brenner, D. and Mak, T. W. Mitochondrial cell death effectors. Curr Opin Cell Biol, *21:* 871-877, 2009.
40. Packham, G. and Stevenson, F. K. Bodyguards and assassins: Bcl-2 family proteins and apoptosis control in chronic lymphocytic leukaemia. Immunology, *114:* 441-449, 2005.
41. Youle, R. J. and Strasser, A. The BCL-2 protein family: opposing activities that mediate cell death. Nat Rev Mol Cell Biol, *9:* 47-59, 2008.
42. Marte, B. M. and Downward, J. PKB/Akt: connecting phosphoinositide 3-kinase to cell survival and beyond. Trends Biochem Sci, *22:* 355-358, 1997.
43. Alessi, D. R. and Cohen, P. Mechanism of activation and function of protein kinase B. Curr Opin Genet Dev, *8:* 55-62, 1998.
44. del Peso, L., Gonzalez-Garcia, M., Page, C., Herrera, R., and Nunez, G. Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. Science, *278:* 687-689, 1997.
45. Datta, S. R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., and Greenberg, M. E. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell, *91:* 231-241, 1997.
46. Dudek, H., Datta, S. R., Franke, T. F., Birnbaum, M. J., Yao, R., Cooper, G. M., Segal, R. A., Kaplan, D. R., and Greenberg, M. E. Regulation of neuronal survival by the serine-threonine protein kinase Akt. Science, *275:* 661-665, 1997.
47. Blume-Jensen, P., Janknecht, R., and Hunter, T. The kit receptor promotes cell survival via activation of PI 3-kinase and subsequent Akt-mediated phosphorylation of Bad on Ser136. Curr Biol, *8:* 779-782, 1998.
48. Gardai, S. J., Hildeman, D. A., Frankel, S. K., Whitlock, B. B., Frasch, S. C., Borregaard, N., Marrack, P., Bratton, D. L., and Henson, P. M. Phosphorylation of Bax Ser184 by Akt regulates its activity and apoptosis in neutrophils. J Biol Chem, *279:* 21085-21095, 2004.
49. van Weeren, P. C., de Bruyn, K. M., de Vries-Smits, A. M., van Lint, J., and Burgering, B. M. Essential role for protein kinase B (PKB) in insulin-induced glycogen synthase kinase 3 inactivation. Characterization of dominant-negative mutant of PKB. J Biol Chem, *273:* 13150-13156, 1998.
50. Cross, D. A., Alessi, D. R., Cohen, P., Andjelkovich, M., and Hemmings, B. A. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature, *378:* 785-789, 1995.
51. Linseman, D. A., Butts, B. D., Precht, T. A., Phelps, R. A., Le, S. S., Laessig, T. A., Bouchard, R. J., Florez-McClure, M. L., and Heidenreich, K. A. Glycogen synthase kinase-3beta phosphorylates Bax and promotes its mitochondrial localization during neuronal apoptosis. J Neurosci, *24:* 9993-10002, 2004.
52. Ding, Q., He, X., Hsu, J. M., Xia, W., Chen, C. T., Li, L. Y., Lee, D. F., Liu, J. C., Zhong, Q., Wang, X., and Hung, M. C. Degradation of Mcl-1 by beta-TrCP mediates glycogen synthase kinase 3-induced tumor suppression and chemosensitization. Mol Cell Biol, *27:* 4006-4017, 2007.

53. Maurer, U., Charvet, C., Wagman, A. S., Dejardin, E., and Green, D. R. Glycogen synthase kinase-3 regulates mitochondrial outer membrane permeabilization and apoptosis by destabilization of MCL-1. Mol Cell, *21:* 749-760, 2006.
54. Adjei, A. A. Novel combinations based on epidermal growth factor receptor inhibition. Clin Cancer Res, *12:* 4446s-4450s, 2006.
55. Arden, K. C. and Biggs, W. H., 3rd Regulation of the FoxO family of transcription factors by phosphatidylinositol-3 kinase-activated signaling. Arch Biochem Biophys, *403:* 292-298, 2002.
56. Brunet, A., Bonni, A., Zigmond, M. J., Lin, M. Z., Juo, P., Hu, L. S., Anderson, M. J., Arden, K. C., Blenis, J., and Greenberg, M. E. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell, *96:* 857-868, 1999.
57. del Peso, L., Gonzalez, V. M., Hernandez, R., Barr, F. G., and Nunez, G. Regulation of the forkhead transcription factor FKHR, but not the PAX3-FKHR fusion protein, by the serine/threonine kinase Akt. Oncogene, *18:* 7328-7333, 1999.
58. Tang, E. D., Nunez, G., Barr, F. G., and Guan, K. L. Negative regulation of the forkhead transcription factor FKHR by Akt. J Biol Chem, *274:* 16741-16746, 1999.
59. Suhara, T., Kim, H. S., Kirshenbaum, L. A., and Walsh, K. Suppression of Akt signaling induces Fas ligand expression: involvement of caspase and Jun kinase activation in Akt-mediated Fas ligand regulation. Mol Cell Biol, *22:* 680-691, 2002.
60. Dijkers, P. F., Medema, R. H., Lammers, J. W., Koenderman, L., and Coffer, P. J. Expression of the pro-apoptotic Bcl-2 family member Bim is regulated by the forkhead transcription factor FKHR-L1. Curr Biol, *10:* 1201-1204, 2000.
61. Willis, S. N. and Adams, J. M. Life in the balance: how BH3-only proteins induce apoptosis. Curr Opin Cell Biol, *17:* 617-625, 2005.
62. Kuwana, T., Bouchier-Hayes, L., Chipuk, J. E., Bonzon, C., Sullivan, B. A., Green, D. R., and Newmeyer, D. D. BH3 domains of BH3-only proteins differentially regulate Bax-mediated mitochondrial membrane permeabilization both directly and indirectly. Mol Cell, *17:* 525-535, 2005.
63. Sonenshein, G. E. Rel/NF-kappa B transcription factors and the control of apoptosis. Semin Cancer Biol, *8:* 113-119, 1997.
64. Bharti, A. C. and Aggarwal, B. B. Nuclear factor-kappa B and cancer: its role in prevention and therapy. Biochem Pharmacol, *64:* 883-888, 2002.
65. Beinke, S. and Ley, S. C. Functions of NF-kappaB1 and NF-kappaB2 in immune cell biology. Biochem J, *382:* 393-409, 2004.
66. Sevilla, L., Zaldumbide, A., Pognonec, P., and Boulukos, K. E. Transcriptional regulation of the bcl-x gene encoding the anti-apoptotic Bcl-xL protein by Ets, Rel/NFkappaB, STAT and AP1 transcription factor families. Histol Histopathol, *16:* 595-601, 2001.
67. Konishi, T., Sasaki, S., Watanabe, T., Kitayama, J., and Nagawa, H. Overexpression of hRFI inhibits 5-fluorouracil-induced apoptosis in colorectal cancer cells via activation of NF-kappaB and upregulation of BCL-2 and BCL-XL. Oncogene, *25:* 3160-3169, 2006.
68. Chen, C., Edelstein, L. C., and Gelinas, C. The Rel/NF-kappaB family directly activates expression of the apoptosis inhibitor Bcl-x(L). Mol Cell Biol, *20:* 2687-2695, 2000.
69. Jones, R. G., Parsons, M., Bonnard, M., Chan, V. S., Yeh, W. C., Woodgett, J. R., and Ohashi, P. S. Protein kinase B regulates T lymphocyte survival, nuclear factor kappaB activation, and Bcl-X(L) levels in vivo. J Exp Med, *191:* 1721-1734, 2000.
70. Hundal, R. S., Gomez-Munoz, A., Kong, J. Y., Salh, B. S., Marotta, A., Duronio, V., and Steinbrecher, U. P. Oxidized low density lipoprotein inhibits macrophage apoptosis by blocking ceramide generation, thereby maintaining protein kinase B activation and Bcl-XL levels. J Biol Chem, *278:* 24399-24408, 2003.
71. Hong, S. Y., Yoon, W. H., Park, J. H., Kang, S. G., Ahn, J. H., and Lee, T. H. Involvement of two NF-kappa B binding elements in tumor necrosis factor alpha -, CD40-, and epstein-barr virus latent membrane protein 1-mediated induction of the cellular inhibitor of apoptosis protein 2 gene. J Biol Chem, *275:* 18022-18028, 2000.
72. Karin, M. and Ben-Neriah, Y. Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. Annu Rev Immunol, *18:* 621-663, 2000.
73. Ozes, O. N., Mayo, L. D., Gustin, J. A., Pfeffer, S. R., Pfeffer, L. M., and Donner, D. B. NF-kappaB activation by tumour necrosis factor requires the Akt serine-threonine kinase. Nature, *401:* 82-85, 1999.
74. Romashkova, J. A. and Makarov, S. S. NF-kappaB is a target of AKT in anti-apoptotic PDGF signalling. Nature, *401:* 86-90, 1999.
75. Mayo, L. D. and Donner, D. B. A phosphatidylinositol 3-kinase/Akt pathway promotes translocation of Mdm2 from the cytoplasm to the nucleus. Proc Natl Acad Sci U S A, *98:* 11598-11603, 2001.
76. Klionsky, D. J., Cregg, J. M., Dunn, W. A., Jr., Emr, S. D., Sakai, Y., Sandoval, I. V., Sibirny, A., Subramani, S., Thumm, M., Veenhuis, M., and Ohsumi, Y. A unified nomenclature for yeast autophagy-related genes. Dev Cell, *5:* 539-545, 2003.

77. Yang, Z. and Klionsky, D. J. Eaten alive: a history of macroautophagy. Nat Cell Biol, *12:* 814-822, 2010.
78. Yang, Z. and Klionsky, D. J. Mammalian autophagy: core molecular machinery and signaling regulation. Curr Opin Cell Biol, *22:* 124-131, 2010.
79. Chen, Y. and Klionsky, D. J. The regulation of autophagy - unanswered questions. J Cell Sci, *124:* 161-170, 2011.
80. Neufeld, T. P. TOR-dependent control of autophagy: biting the hand that feeds. Curr Opin Cell Biol, *22:* 157-168, 2010.
81. Maiuri, M. C., Zalckvar, E., Kimchi, A., and Kroemer, G. Self-eating and self-killing: crosstalk between autophagy and apoptosis. Nat Rev Mol Cell Biol, *8:* 741-752, 2007.
82. Gump, J. M. and Thorburn, A. Autophagy and apoptosis: what is the connection? Trends Cell Biol, *21:* 387-392, 2011.
83. Eisenberg-Lerner, A., Bialik, S., Simon, H. U., and Kimchi, A. Life and death partners: apoptosis, autophagy and the cross-talk between them. Cell Death Differ, *16:* 966-975, 2009.
84. Jemal, A., Siegel, R., Xu, J., and Ward, E. Cancer Statistics, 2010. CA Cancer J Clin, 2010.
85. Vandenbroucke, E., De Ryck, F., Surmont, V., and van Meerbeeck, J. P. What is the role for surgery in patients with stage III non-small cell lung cancer? Curr Opin Pulm Med, *15:* 295-302, 2009.
86. Robinson, C. G. and Bradley, J. D. The treatment of early-stage disease. Semin Radiat Oncol, *20:* 178-185, 2010.
87. Molina, J. R., Adjei, A. A., and Jett, J. R. Advances in chemotherapy of non-small cell lung cancer. Chest, *130:* 1211-1219, 2006.
88. Jackman, D. M. and Johnson, B. E. Small-cell lung cancer. Lancet, *366:* 1385-1396, 2005.
89. Demedts, I. K., Vermaelen, K. Y., and van Meerbeeck, J. P. Treatment of extensive-stage small cell lung carcinoma: current status and future prospects. Eur Respir J, *35:* 202-215, 2010.
90. Sculier, J. P., Berghmans, T., and Meert, A. P. Update in lung cancer and mesothelioma 2009. Am J Respir Crit Care Med, *181:* 773-781, 2010.
91. Govindan, R., Page, N., Morgensztern, D., Read, W., Tierney, R., Vlahiotis, A., Spitznagel, E. L., and Piccirillo, J. Changing epidemiology of small-cell lung cancer in the United States over the last 30 years: analysis of the surveillance, epidemiologic, and end results database. J Clin Oncol, *24:* 4539-4544, 2006.
92. Cooper, S. and Spiro, S. G. Small cell lung cancer: treatment review. Respirology, *11:* 241-248, 2006.
93. Barnard, W. The nature of the "oat-celled sarcoma" of the mediastinum. J Pathol, *29:* 241-244, 1926.
94. Travis, W. D. Advances in neuroendocrine lung tumors. Ann Oncol, *21 Suppl 7:* vii65-71, 2010.
95. Irshad, A. and Ravenel, J. G. Imaging of small-cell lung cancer. Curr Probl Diagn Radiol, *33:* 200-211, 2004.
96. Planchard, D. and Le Pechoux, C. Small cell lung cancer: new clinical recommendations and current status of biomarker assessment. Eur J Cancer, *47 Suppl 3:* S272-283, 2011.
97. Janne, P. A. and Skarin, A. T. Concurrent docetaxel and thoracic radiation in non-small-cell lung cancer. Clin Lung Cancer, *3 Suppl 2:* S37-41, 2002.
98. Shepherd, F. A., Crowley, J., Van Houtte, P., Postmus, P. E., Carney, D., Chansky, K., Shaikh, Z., and Goldstraw, P. The International Association for the Study of Lung Cancer lung cancer staging project: proposals regarding the clinical staging of small cell lung cancer in the forthcoming (seventh) edition of the tumor, node, metastasis classification for lung cancer. J Thorac Oncol, *2:* 1067-1077, 2007.
99. Rodriguez, E. and Lilenbaum, R. C. Small cell lung cancer: past, present, and future. Curr Oncol Rep, *12:* 327-334, 2010.
100. Hurwitz, J. L., McCoy, F., Scullin, P., and Fennell, D. A. New advances in the second-line treatment of small cell lung cancer. Oncologist, *14:* 986-994, 2009.
101. Stovold, R., Blackhall, F., Meredith, S., Hou, J., Dive, C., and White, A. Biomarkers for small cell lung cancer: Neuroendocrine, epithelial and circulating tumour cells. Lung Cancer, *76:* 263-268, 2012.
102. Meuwissen, R., Linn, S. C., Linnoila, R. I., Zevenhoven, J., Mooi, W. J., and Berns, A. Induction of small cell lung cancer by somatic inactivation of both Trp53 and Rb1 in a conditional mouse model. Cancer Cell, *4:* 181-189, 2003.
103. Park, K. S., Liang, M. C., Raiser, D. M., Zamponi, R., Roach, R. R., Curtis, S. J., Walton, Z., Schaffer, B. E., Roake, C. M., Zmoos, A. F., Kriegel, C., Wong, K. K., Sage, J., and Kim, C. F. Characterization of the cell of origin for small cell lung cancer. Cell Cycle, *10:* 2806-2815, 2011.
104. Sutherland, K. D., Proost, N., Brouns, I., Adriaensen, D., Song, J. Y., and Berns, A. Cell of origin of small cell lung cancer: inactivation of Trp53 and Rb1 in distinct cell types of adult mouse lung. Cancer Cell, *19:* 754-764, 2011.
105. D'Angelo, S. P. and Pietanza, M. C. The molecular pathogenesis of small cell lung cancer. Cancer Biol Ther, *10:* 1-10, 2010.
106. Heasley, L. E. Autocrine and paracrine signaling through neuropeptide receptors in human cancer. Oncogene, *20:* 1563-1569, 2001.
107. Rosti, G., Bevilacqua, G., Bidoli, P., Portalone, L., Santo, A., and Genestreti, G. Small cell lung cancer. Ann Oncol, *17 Suppl 2:* ii5-ii10, 2006.

108. Wistuba, II, Gazdar, A. F., and Minna, J. D. Molecular genetics of small cell lung carcinoma. Semin Oncol, *28:* 3-13, 2001.
109. Sato, M., Shames, D. S., Gazdar, A. F., and Minna, J. D. A translational view of the molecular pathogenesis of lung cancer. J Thorac Oncol, *2:* 327-343, 2007.
110. Sattler, M. and Salgia, R. Molecular and cellular biology of small cell lung cancer. Semin Oncol, *30:* 57-71, 2003.
111. Ishida, T., Chada, S., Stipanov, M., Nadaf, S., Ciernik, F. I., Gabrilovich, D. I., and Carbone, D. P. Dendritic cells transduced with wild-type p53 gene elicit potent anti-tumour immune responses. Clin Exp Immunol, *117:* 244-251, 1999.
112. Modi, S., Kubo, A., Oie, H., Coxon, A. B., Rehmatulla, A., and Kaye, F. J. Protein expression of the RB-related gene family and SV40 large T antigen in mesothelioma and lung cancer. Oncogene, *19:* 4632-4639, 2000.
113. Sekido, Y., Fong, K. M., and Minna, J. D. Molecular genetics of lung cancer. Annu Rev Med, *54:* 73-87, 2003.
114. Ronnstrand, L. Signal transduction via the stem cell factor receptor/c-Kit. Cell Mol Life Sci, *61:* 2535-2548, 2004.
115. Sekido, Y., Obata, Y., Ueda, R., Hida, T., Suyama, M., Shimokata, K., Ariyoshi, Y., and Takahashi, T. Preferential expression of c-kit protooncogene transcripts in small cell lung cancer. Cancer Res, *51:* 2416-2419, 1991.
116. Rygaard, K., Nakamura, T., and Spang-Thomsen, M. Expression of the proto-oncogenes c-met and c-kit and their ligands, hepatocyte growth factor/scatter factor and stem cell factor, in SCLC cell lines and xenografts. Br J Cancer, *67:* 37-46, 1993.
117. Hibi, K., Takahashi, T., Sekido, Y., Ueda, R., Hida, T., Ariyoshi, Y., Takagi, H., and Takahashi, T. Coexpression of the stem cell factor and the c-kit genes in small-cell lung cancer. Oncogene, *6:* 2291-2296, 1991.
118. Fischer, B., Marinov, M., and Arcaro, A. Targeting receptor tyrosine kinase signalling in small cell lung cancer (SCLC): what have we learned so far? Cancer Treat Rev, *33:* 391-406, 2007.
119. Suyama, H., Igishi, T., Ueda, Y., Shigeoka, Y., Kodani, M., Morita, M., Takeda, K., Sumikawa, T., Nakazaki, H., Matsunami, K., Matsumoto, S., and Shimizu, E. Imatinib mesylate (STI571) enhances amrubicin-induced cytotoxic activity through inhibition of the phosphatidylinositol 3-kinase/Akt pathway in small cell lung cancer cells. Oncol Rep, *23:* 217-222, 2010.
120. Decaudin, D., de Cremoux, P., Sastre, X., Judde, J. G., Nemati, F., Tran-Perennou, C., Freneaux, P., Livartowski, A., Pouillart, P., and Poupon, M. F. In vivo efficacy of STI571 in xenografted human small cell lung cancer alone or combined with chemotherapy. Int J Cancer, *113:* 849-856, 2005.
121. Warshamana-Greene, G. S., Litz, J., Buchdunger, E., Hofmann, F., Garcia-Echeverria, C., and Krystal, G. W. The insulin-like growth factor-I (IGF-I) receptor kinase inhibitor NVP-ADW742, in combination with STI571, delineates a spectrum of dependence of small cell lung cancer on IGF-I and stem cell factor signaling. Mol Cancer Ther, *3:* 527-535, 2004.
122. Krystal, G. W., Honsawek, S., Litz, J., and Buchdunger, E. The selective tyrosine kinase inhibitor STI571 inhibits small cell lung cancer growth. Clin Cancer Res, *6:* 3319-3326, 2000.
123. Tsurutani, J., West, K. A., Sayyah, J., Gills, J. J., and Dennis, P. A. Inhibition of the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin pathway but not the MEK/ERK pathway attenuates laminin-mediated small cell lung cancer cellular survival and resistance to imatinib mesylate or chemotherapy. Cancer Res, *65:* 8423-8432, 2005.
124. Schneider, B. J., Kalemkerian, G. P., Ramnath, N., Kraut, M. J., Wozniak, A. J., Worden, F. P., Ruckdeschel, J. C., Zhang, X., Chen, W., and Gadgeel, S. M. Phase II trial of imatinib maintenance therapy after irinotecan and cisplatin in patients with c-Kit-positive, extensive-stage small-cell lung cancer. Clin Lung Cancer, *11:* 223-227, 2010.
125. Spigel, D. R., Hainsworth, J. D., Simons, L., Meng, C., Burris, H. A., 3rd, Yardley, D. A., Grapski, R., Schreeder, M., Mallidi, P. V., and Greco, F. A. Irinotecan, carboplatin, and imatinib in untreated extensive-stage small-cell lung cancer: a phase II trial of the Minnie Pearl Cancer Research Network. J Thorac Oncol, *2:* 854-861, 2007.
126. Dy, G. K., Miller, A. A., Mandrekar, S. J., Aubry, M. C., Langdon, R. M., Jr., Morton, R. F., Schild, S. E., Jett, J. R., and Adjei, A. A. A phase II trial of imatinib (ST1571) in patients with c-kit expressing relapsed small-cell lung cancer: a CALGB and NCCTG study. Ann Oncol, *16:* 1811-1816, 2005.
127. Johnson, B. E., Fischer, T., Fischer, B., Dunlop, D., Rischin, D., Silberman, S., Kowalski, M. O., Sayles, D., Dimitrijevic, S., Fletcher, C., Hornick, J., Salgia, R., and Le Chevalier, T. Phase II study of imatinib in patients with small cell lung cancer. Clin Cancer Res, *9:* 5880-5887, 2003.
128. Niklinski, J. and Furman, M. Clinical tumour markers in lung cancer. Eur J Cancer Prev, *4:* 129-138, 1995.

129. Quinn, K. A., Treston, A. M., Unsworth, E. J., Miller, M. J., Vos, M., Grimley, C., Battey, J., Mulshine, J. L., and Cuttitta, F. Insulin-like growth factor expression in human cancer cell lines. J Biol Chem, *271:* 11477-11483, 1996.
130. Nakanishi, Y., Mulshine, J. L., Kasprzyk, P. G., Natale, R. B., Maneckjee, R., Avis, I., Treston, A. M., Gazdar, A. F., Minna, J. D., and Cuttitta, F. Insulin-like growth factor-I can mediate autocrine proliferation of human small cell lung cancer cell lines in vitro. J Clin Invest, *82:* 354-359, 1988.
131. Rotsch, M., Maasberg, M., Erbil, C., Jaques, G., Worsch, U., and Havemann, K. Characterization of insulin-like growth factor I receptors and growth effects in human lung cancer cell lines. J Cancer Res Clin Oncol, *118:* 502-508, 1992.
132. Macaulay, V. M., Everard, M. J., Teale, J. D., Trott, P. A., Van Wyk, J. J., Smith, I. E., and Millar, J. L. Autocrine function for insulin-like growth factor I in human small cell lung cancer cell lines and fresh tumor cells. Cancer Res, *50:* 2511-2517, 1990.
133. Warshamana-Greene, G. S., Litz, J., Buchdunger, E., Garcia-Echeverria, C., Hofmann, F., and Krystal, G. W. The insulin-like growth factor-I receptor kinase inhibitor, NVP-ADW742, sensitizes small cell lung cancer cell lines to the effects of chemotherapy. Clin Cancer Res, *11:* 1563-1571, 2005.
134. Yeh, J., Litz, J., Hauck, P., Ludwig, D. L., and Krystal, G. W. Selective inhibition of SCLC growth by the A12 anti-IGF-1R monoclonal antibody correlates with inhibition of Akt. Lung Cancer, *60:* 166-174, 2008.
135. Maulik, G., Kijima, T., Ma, P. C., Ghosh, S. K., Lin, J., Shapiro, G. I., Schaefer, E., Tibaldi, E., Johnson, B. E., and Salgia, R. Modulation of the c-Met/hepatocyte growth factor pathway in small cell lung cancer. Clin Cancer Res, *8:* 620-627, 2002.
136. Maulik, G., Madhiwala, P., Brooks, S., Ma, P. C., Kijima, T., Tibaldi, E. V., Schaefer, E., Parmar, K., and Salgia, R. Activated c-Met signals through PI3K with dramatic effects on cytoskeletal functions in small cell lung cancer. J Cell Mol Med, *6:* 539-553, 2002.
137. Jafri, N. F., Ma, P. C., Maulik, G., and Salgia, R. Mechanisms of metastasis as related to receptor tyrosine kinases in small-cell lung cancer. J Environ Pathol Toxicol Oncol, *22:* 147-165, 2003.
138. Ma, P. C., Kijima, T., Maulik, G., Fox, E. A., Sattler, M., Griffin, J. D., Johnson, B. E., and Salgia, R. c-MET mutational analysis in small cell lung cancer: novel juxtamembrane domain mutations regulating cytoskeletal functions. Cancer Res, *63:* 6272-6281, 2003.
139. Tanno, S., Ohsaki, Y., Nakanishi, K., Toyoshima, E., and Kikuchi, K. Human small cell lung cancer cells express functional VEGF receptors, VEGFR-2 and VEGFR-3. Lung Cancer, *46:* 11-19, 2004.
140. Lund, E. L., Thorsen, C., Pedersen, M. W., Junker, N., and Kristjansen, P. E. Relationship between vessel density and expression of vascular endothelial growth factor and basic fibroblast growth factor in small cell lung cancer in vivo and in vitro. Clin Cancer Res, *6:* 4287-4291, 2000.
141. Takigawa, N., Segawa, Y., Fujimoto, N., Hotta, K., and Eguchi, K. Elevated vascular endothelial growth factor levels in sera of patients with lung cancer. Anticancer Res, *18:* 1251-1254, 1998.
142. Mall, J. W., Schwenk, W., Philipp, A. W., Meyer-Kipker, C., Mall, W., Muller, J., and Pollmann, C. Serum vascular endothelial growth factor levels correlate better with tumour stage in small cell lung cancer than albumin, neuron-specific enolase or lactate dehydrogenase. Respirology, *7:* 99-102, 2002.
143. Salven, P., Ruotsalainen, T., Mattson, K., and Joensuu, H. High pre-treatment serum level of vascular endothelial growth factor (VEGF) is associated with poor outcome in small-cell lung cancer. Int J Cancer, *79:* 144-146, 1998.
144. Hasegawa, Y., Takanashi, S., Okudera, K., Kumagai, M., Hayashi, A., Morimoto, T., and Okumura, K. Vascular endothelial growth factor level as a prognostic determinant of small cell lung cancer in Japanese patients. Intern Med, *44:* 26-34, 2005.
145. Stefanou, D., Batistatou, A., Arkoumani, E., Ntzani, E., and Agnantis, N. J. Expression of vascular endothelial growth factor (VEGF) and association with microvessel density in small-cell and non-small-cell lung carcinomas. Histol Histopathol, *19:* 37-42, 2004.
146. Ruotsalainen, T., Joensuu, H., Mattson, K., and Salven, P. High pretreatment serum concentration of basic fibroblast growth factor is a predictor of poor prognosis in small cell lung cancer. Cancer Epidemiol Biomarkers Prev, *11:* 1492-1495, 2002.
147. Pardo, O. E., Arcaro, A., Salerno, G., Tetley, T. D., Valovka, T., Gout, I., and Seckl, M. J. Novel cross talk between MEK and S6K2 in FGF-2 induced proliferation of SCLC cells. Oncogene, *20:* 7658-7667, 2001.
148. Pardo, O. E., Wellbrock, C., Khanzada, U. K., Aubert, M., Arozarena, I., Davidson, S., Bowen, F., Parker, P. J., Filonenko, V. V., Gout, I. T., Sebire, N., Marais, R., Downward, J., and Seckl, M. J. FGF-2 protects small cell lung cancer cells from apoptosis through a complex involving PKCepsilon, B-Raf and S6K2. Embo J, *25:* 3078-3088, 2006.
149. Pardo, O. E., Arcaro, A., Salerno, G., Raguz, S., Downward, J., and Seckl, M. J. Fibroblast growth factor-2 induces translational regulation of Bcl-XL and Bcl-2 via a MEK-dependent pathway: correlation with resistance to etoposide-induced apoptosis. J Biol Chem, *277:* 12040-12046, 2002.

150. Pardo, O. E., Lesay, A., Arcaro, A., Lopes, R., Ng, B. L., Warne, P. H., McNeish, I. A., Tetley, T. D., Lemoine, N. R., Mehmet, H., Seckl, M. J., and Downward, J. Fibroblast growth factor 2-mediated translational control of IAPs blocks mitochondrial release of Smac/DIABLO and apoptosis in small cell lung cancer cells. Mol Cell Biol, *23:* 7600-7610, 2003.
151. Pardo, O. E., Latigo, J., Jeffery, R. E., Nye, E., Poulsom, R., Spencer-Dene, B., Lemoine, N. R., Stamp, G. W., Aboagye, E. O., and Seckl, M. J. The fibroblast growth factor receptor inhibitor PD173074 blocks small cell lung cancer growth in vitro and in vivo. Cancer Res, *69:* 8645-8651, 2009.
152. Kaiser, U., Schilli, M., Haag, U., Neumann, K., Kreipe, H., Kogan, E., and Havemann, K. Expression of bcl-2--protein in small cell lung cancer. Lung Cancer, *15:* 31-40, 1996.
153. Ben-Ezra, J. M., Kornstein, M. J., Grimes, M. M., and Krystal, G. Small cell carcinomas of the lung express the Bcl-2 protein. Am J Pathol, *145:* 1036-1040, 1994.
154. Higashiyama, M., Doi, O., Kodama, K., Yokouchi, H., and Tateishi, R. High prevalence of bcl-2 oncoprotein expression in small cell lung cancer. Anticancer Res, *15:* 503-505, 1995.
155. Jiang, S. X., Sato, Y., Kuwao, S., and Kameya, T. Expression of bcl-2 oncogene protein is prevalent in small cell lung carcinomas. J Pathol, *177:* 135-138, 1995.
156. Sethi, T., Rintoul, R. C., Moore, S. M., MacKinnon, A. C., Salter, D., Choo, C., Chilvers, E. R., Dransfield, I., Donnelly, S. C., Strieter, R., and Haslett, C. Extracellular matrix proteins protect small cell lung cancer cells against apoptosis: a mechanism for small cell lung cancer growth and drug resistance in vivo. Nat Med, *5:* 662-668, 1999.
157. Sartorius, U. A. and Krammer, P. H. Upregulation of Bcl-2 is involved in the mediation of chemotherapy resistance in human small cell lung cancer cell lines. Int J Cancer, *97:* 584-592, 2002.
158. Takayama, K., Ogata, K., Nakanishi, Y., Yatsunami, J., Kawasaki, M., and Hara, N. Bcl-2 expression as a predictor of chemosensitivities and survival in small cell lung cancer. Cancer J Sci Am, *2:* 212-216, 1996.
159. Zereu, M., Vinholes, J. J., and Zettler, C. G. p53 and Bcl-2 protein expression and its relationship with prognosis in small-cell lung cancer. Clin Lung Cancer, *4:* 298-302, 2003.
160. Stefanaki, K., Rontogiannis, D., Vamvouka, C., Bolioti, S., Chaniotis, V., Sotsiou, F., Vlychou, M., Delidis, G., Kakolyris, S., Georgoulias, V., and Kanavaros, P. Immunohistochemical detection of bcl2, p53, mdm2 and p21/waf1 proteins in small-cell lung carcinomas. Anticancer Res, *18:* 1689-1695, 1998.
161. Ohmori, T., Podack, E. R., Nishio, K., Takahashi, M., Miyahara, Y., Takeda, Y., Kubota, N., Funayama, Y., Ogasawara, H., Ohira, T., and et al. Apoptosis of lung cancer cells caused by some anti-cancer agents (MMC, CPT-11, ADM) is inhibited by bcl-2. Biochem Biophys Res Commun, *192:* 30-36, 1993.
162. Zangemeister-Wittke, U., Schenker, T., Luedke, G. H., and Stahel, R. A. Synergistic cytotoxicity of bcl-2 antisense oligodeoxynucleotides and etoposide, doxorubicin and cisplatin on small-cell lung cancer cell lines. Br J Cancer, *78:* 1035-1042, 1998.
163. Ziegler, A., Luedke, G. H., Fabbro, D., Altmann, K. H., Stahel, R. A., and Zangemeister-Wittke, U. Induction of apoptosis in small-cell lung cancer cells by an antisense oligodeoxynucleotide targeting the Bcl-2 coding sequence. J Natl Cancer Inst, *89:* 1027-1036, 1997.
164. Bloom, J., Amador, V., Bartolini, F., DeMartino, G., and Pagano, M. Proteasome-mediated degradation of p21 via N-terminal ubiquitinylation. Cell, *115:* 71-82, 2003.
165. Martin, B., Paesmans, M., Berghmans, T., Branle, F., Ghisdal, L., Mascaux, C., Meert, A. P., Steels, E., Vallot, F., Verdebout, J. M., Lafitte, J. J., and Sculier, J. P. Role of Bcl-2 as a prognostic factor for survival in lung cancer: a systematic review of the literature with meta-analysis. Br J Cancer, *89:* 55-64, 2003.
166. Paik, P. K., Rudin, C. M., Pietanza, M. C., Brown, A., Rizvi, N. A., Takebe, N., Travis, W., James, L., Ginsberg, M. S., Juergens, R., Markus, S., Tyson, L., Subzwari, S., Kris, M. G., and Krug, L. M. A phase II study of obatoclax mesylate, a Bcl-2 antagonist, plus topotecan in relapsed small cell lung cancer. Lung Cancer, *74:* 481-485, 2011.
167. Chiappori, A. A., Schreeder, M. T., Moezi, M. M., Stephenson, J. J., Blakely, J., Salgia, R., Chu, Q. S., Ross, H. J., Subramaniam, D. S., Schnyder, J., and Berger, M. S. A phase I trial of pan-Bcl-2 antagonist obatoclax administered as a 3-h or a 24-h infusion in combination with carboplatin and etoposide in patients with extensive-stage small cell lung cancer. Br J Cancer, *106:* 839-845, 2012.
168. Dean, E. J., Cummings, J., Roulston, A., Berger, M., Ranson, M., Blackhall, F., and Dive, C. Optimization of circulating biomarkers of obatoclax-induced cell death in patients with small cell lung cancer. Neoplasia, *13:* 339-347, 2011.
169. Xu, H. and Krystal, G. W. Actinomycin D decreases Mcl-1 expression and acts synergistically with ABT-737 against small cell lung cancer cell lines. Clin Cancer Res, *16:* 4392-4400, 2010.
170. Hauck, P., Chao, B. H., Litz, J., and Krystal, G. W. Alterations in the Noxa/Mcl-1 axis determine sensitivity of small cell lung cancer to the BH3 mimetic ABT-737. Mol Cancer Ther, *8:* 883-892, 2009.
171. Micha, D., Cummings, J., Shoemaker, A., Elmore, S., Foster, K., Greaves, M., Ward, T., Rosenberg, S., Dive, C., and Simpson, K. Circulating biomarkers of cell death after treatment with the BH-3 mimetic ABT-737 in a preclinical model of small-cell lung cancer. Clin Cancer Res, *14:* 7304-7310, 2008.

172. Hann, C. L., Daniel, V. C., Sugar, E. A., Dobromilskaya, I., Murphy, S. C., Cope, L., Lin, X., Hierman, J. S., Wilburn, D. L., Watkins, D. N., and Rudin, C. M. Therapeutic efficacy of ABT-737, a selective inhibitor of BCL-2, in small cell lung cancer. Cancer Res, *68:* 2321-2328, 2008.
173. Tahir, S. K., Yang, X., Anderson, M. G., Morgan-Lappe, S. E., Sarthy, A. V., Chen, J., Warner, R. B., Ng, S. C., Fesik, S. W., Elmore, S. W., Rosenberg, S. H., and Tse, C. Influence of Bcl-2 family members on the cellular response of small-cell lung cancer cell lines to ABT-737. Cancer Res, *67:* 1176-1183, 2007.
174. Shibata, T., Kokubu, A., Tsuta, K., and Hirohashi, S. Oncogenic mutation of PIK3CA in small cell lung carcinoma: a potential therapeutic target pathway for chemotherapy-resistant lung cancer. Cancer Lett, *283:* 203-211, 2009.
175. Yamamoto, H., Shigematsu, H., Nomura, M., Lockwood, W. W., Sato, M., Okumura, N., Soh, J., Suzuki, M., Wistuba, II, Fong, K. M., Lee, H., Toyooka, S., Date, H., Lam, W. L., Minna, J. D., and Gazdar, A. F. PIK3CA mutations and copy number gains in human lung cancers. Cancer Res, *68:* 6913-6921, 2008.
176. Massion, P. P., Taflan, P. M., Shyr, Y., Rahman, S. M., Yildiz, P., Shakthour, B., Edgerton, M. E., Ninan, M., Andersen, J. J., and Gonzalez, A. L. Early involvement of the phosphatidylinositol 3-kinase/Akt pathway in lung cancer progression. Am J Respir Crit Care Med, *170:* 1088-1094, 2004.
177. Voortman, J., Lee, J. H., Killian, J. K., Suuriniemi, M., Wang, Y., Lucchi, M., Smith, W. I., Jr., Meltzer, P., and Giaccone, G. Array comparative genomic hybridization-based characterization of genetic alterations in pulmonary neuroendocrine tumors. Proc Natl Acad Sci U S A, 2010.
178. Forgacs, E., Biesterveld, E. J., Sekido, Y., Fong, K., Muneer, S., Wistuba, II, Milchgrub, S., Brezinschek, R., Virmani, A., Gazdar, A. F., and Minna, J. D. Mutation analysis of the PTEN/MMAC1 gene in lung cancer. Oncogene, *17:* 1557-1565, 1998.
179. Forgacs, E., Zochbauer-Muller, S., Olah, E., and Minna, J. D. Molecular genetic abnormalities in the pathogenesis of human lung cancer. Pathol Oncol Res, *7:* 6-13, 2001.
180. Yokomizo, A., Tindall, D. J., Drabkin, H., Gemmill, R., Franklin, W., Yang, P., Sugio, K., Smith, D. I., and Liu, W. PTEN/MMAC1 mutations identified in small cell, but not in non-small cell lung cancers. Oncogene, *17:* 475-479, 1998.
181. Moore, S. M., Rintoul, R. C., Walker, T. R., Chilvers, E. R., Haslett, C., and Sethi, T. The presence of a constitutively active phosphoinositide 3-kinase in small cell lung cancer cells mediates anchorage-independent proliferation via a protein kinase B and p70s6k-dependent pathway. Cancer Res, *58:* 5239-5247, 1998.
182. Arcaro, A., Khanzada, U. K., Vanhaesebroeck, B., Tetley, T. D., Waterfield, M. D., and Seckl, M. J. Two distinct phosphoinositide 3-kinases mediate polypeptide growth factor-stimulated PKB activation. Embo J, *21:* 5097-5108, 2002.
183. Blackhall, F. H., Pintilie, M., Michael, M., Leighl, N., Feld, R., Tsao, M. S., and Shepherd, F. A. Expression and prognostic significance of kit, protein kinase B, and mitogen-activated protein kinase in patients with small cell lung cancer. Clin Cancer Res, *9:* 2241-2247, 2003.
184. West, K. A., Brognard, J., Clark, A. S., Linnoila, I. R., Yang, X., Swain, S. M., Harris, C., Belinsky, S., and Dennis, P. A. Rapid Akt activation by nicotine and a tobacco carcinogen modulates the phenotype of normal human airway epithelial cells. J Clin Invest, *111:* 81-90, 2003.
185. Kraus, A. C., Ferber, I., Bachmann, S. O., Specht, H., Wimmel, A., Gross, M. W., Schlegel, J., Suske, G., and Schuermann, M. In vitro chemo- and radio-resistance in small cell lung cancer correlates with cell adhesion and constitutive activation of AKT and MAP kinase pathways. Oncogene, *21:* 8683-8695, 2002.
186. Belyanskaya, L. L., Hopkins-Donaldson, S., Kurtz, S., Simoes-Wust, A. P., Yousefi, S., Simon, H. U., Stahel, R., and Zangemeister-Wittke, U. Cisplatin activates Akt in small cell lung cancer cells and attenuates apoptosis by survivin upregulation. Int J Cancer, *117:* 755-763, 2005.
187. Krystal, G. W., Sulanke, G., and Litz, J. Inhibition of phosphatidylinositol 3-kinase-Akt signaling blocks growth, promotes apoptosis, and enhances sensitivity of small cell lung cancer cells to chemotherapy. Mol Cancer Ther, *1:* 913-922, 2002.
188. Ma, P. C., Tretiakova, M. S., Nallasura, V., Jagadeeswaran, R., Husain, A. N., and Salgia, R. Downstream signalling and specific inhibition of c-MET/HGF pathway in small cell lung cancer: implications for tumour invasion. Br J Cancer, *97:* 368-377, 2007.
189. Wang, W. L., Healy, M. E., Sattler, M., Verma, S., Lin, J., Maulik, G., Stiles, C. D., Griffin, J. D., Johnson, B. E., and Salgia, R. Growth inhibition and modulation of kinase pathways of small cell lung cancer cell lines by the novel tyrosine kinase inhibitor STI 571. Oncogene, *19:* 3521-3528, 2000.
190. Chen, Y. L., Law, P. Y., and Loh, H. H. Inhibition of akt/protein kinase B signaling by naltrindole in small cell lung cancer cells. Cancer Res, *64:* 8723-8730, 2004.
191. Carlisle, D. L., Liu, X., Hopkins, T. M., Swick, M. C., Dhir, R., and Siegfried, J. M. Nicotine activates cell-signaling pathways through muscle-type and neuronal nicotinic acetylcholine receptors in non-small cell lung cancer cells. Pulm Pharmacol Ther, 2006.

192. Tsurutani, J., Castillo, S. S., Brognard, J., Granville, C. A., Zhang, C., Gills, J. J., Sayyah, J., and Dennis, P. A. Tobacco components stimulate Akt-dependent proliferation and NFkappaB-dependent survival in lung cancer cells. Carcinogenesis, *26:* 1182-1195, 2005.
193. Gustafson, A. M., Soldi, R., Anderlind, C., Scholand, M. B., Qian, J., Zhang, X., Cooper, K., Walker, D., McWilliams, A., Liu, G., Szabo, E., Brody, J., Massion, P. P., Lenburg, M. E., Lam, S., Bild, A. H., and Spira, A. Airway PI3K pathway activation is an early and reversible event in lung cancer development. Sci Transl Med, *2:* 26ra25, 2010.
194. Sun, X., Ritzenthaler, J. D., Zhong, X., Zheng, Y., Roman, J., and Han, S. Nicotine stimulates PPARbeta/delta expression in human lung carcinoma cells through activation of PI3K/mTOR and suppression of AP-2alpha. Cancer Res, *69:* 6445-6453, 2009.
195. Xin, M. and Deng, X. Nicotine inactivation of the proapoptotic function of Bax through phosphorylation. J Biol Chem, *280:* 10781-10789, 2005.
196. Jin, Z., Gao, F., Flagg, T., and Deng, X. Nicotine induces multi-site phosphorylation of Bad in association with suppression of apoptosis. J Biol Chem, *279:* 23837-23844, 2004.
197. Brodeur, G. M. Neuroblastoma: biological insights into a clinical enigma. Nat Rev Cancer, *3:* 203-216, 2003.
198. Maris, J. M., Hogarty, M. D., Bagatell, R., and Cohn, S. L. Neuroblastoma. Lancet, *369:* 2106-2120, 2007.
199. London, W. B., Castleberry, R. P., Matthay, K. K., Look, A. T., Seeger, R. C., Shimada, H., Thorner, P., Brodeur, G., Maris, J. M., Reynolds, C. P., and Cohn, S. L. Evidence for an age cutoff greater than 365 days for neuroblastoma risk group stratification in the Children's Oncology Group. J Clin Oncol, *23:* 6459-6465, 2005.
200. Hoehner, J. C., Gestblom, C., Hedborg, F., Sandstedt, B., Olsen, L., and Pahlman, S. A developmental model of neuroblastoma: differentiating stroma-poor tumors' progress along an extra-adrenal chromaffin lineage. Lab Invest, *75:* 659-675, 1996.
201. Lonergan, G. J., Schwab, C. M., Suarez, E. S., and Carlson, C. L. Neuroblastoma, ganglioneuroblastoma, and ganglioneuroma: radiologic-pathologic correlation. Radiographics, *22:* 911-934, 2002.
202. Carlsen, N. L. How frequent is spontaneous remission of neuroblastomas? Implications for screening. Br J Cancer, *61:* 441-446, 1990.
203. Yamamoto, K., Hanada, R., Kikuchi, A., Ichikawa, M., Aihara, T., Oguma, E., Moritani, T., Shimanuki, Y., Tanimura, M., and Hayashi, Y. Spontaneous regression of localized neuroblastoma detected by mass screening. J Clin Oncol, *16:* 1265-1269, 1998.
204. De Bernardi, B., Nicolas, B., Boni, L., Indolfi, P., Carli, M., Cordero Di Montezemolo, L., Donfrancesco, A., Pession, A., Provenzi, M., di Cataldo, A., Rizzo, A., Tonini, G. P., Dallorso, S., Conte, M., Gambini, C., Garaventa, A., Bonetti, F., Zanazzo, A., D'Angelo, P., and Bruzzi, P. Disseminated neuroblastoma in children older than one year at diagnosis: comparable results with three consecutive high-dose protocols adopted by the Italian Co-Operative Group for Neuroblastoma. J Clin Oncol, *21:* 1592-1601, 2003.
205. Matthay, K. K., Villablanca, J. G., Seeger, R. C., Stram, D. O., Harris, R. E., Ramsay, N. K., Swift, P., Shimada, H., Black, C. T., Brodeur, G. M., Gerbing, R. B., and Reynolds, C. P. Treatment of high-risk neuroblastoma with intensive chemotherapy, radiotherapy, autologous bone marrow transplantation, and 13-cis-retinoic acid. Children's Cancer Group. N Engl J Med, *341:* 1165-1173, 1999.
206. Brodeur, G. M., Pritchard, J., Berthold, F., Carlsen, N. L., Castel, V., Castelberry, R. P., De Bernardi, B., Evans, A. E., Favrot, M., Hedborg, F., and et al. Revisions of the international criteria for neuroblastoma diagnosis, staging, and response to treatment. J Clin Oncol, *11:* 1466-1477, 1993.
207. Monclair, T., Brodeur, G. M., Ambros, P. F., Brisse, H. J., Cecchetto, G., Holmes, K., Kaneko, M., London, W. B., Matthay, K. K., Nuchtern, J. G., von Schweinitz, D., Simon, T., Cohn, S. L., and Pearson, A. D. The International Neuroblastoma Risk Group (INRG) staging system: an INRG Task Force report. J Clin Oncol, *27:* 298-303, 2009.
208. Nitschke, R., Smith, E. I., Shochat, S., Altshuler, G., Travers, H., Shuster, J. J., Hayes, F. A., Patterson, R., and McWilliams, N. Localized neuroblastoma treated by surgery: a Pediatric Oncology Group Study. J Clin Oncol, *6:* 1271-1279, 1988.
209. Park, J. R., Eggert, A., and Caron, H. Neuroblastoma: biology, prognosis, and treatment. Pediatr Clin North Am, *55:* 97-120, x, 2008.
210. Maris, J. M. Recent advances in neuroblastoma. N Engl J Med, *362:* 2202-2211, 2010.
211. Modak, S. and Cheung, N. K. Neuroblastoma: Therapeutic strategies for a clinical enigma. Cancer Treat Rev, *36:* 307-317, 2010.
212. Pearson, A. D., Pinkerton, C. R., Lewis, I. J., Imeson, J., Ellershaw, C., and Machin, D. High-dose rapid and standard induction chemotherapy for patients aged over 1 year with stage 4 neuroblastoma: a randomised trial. Lancet Oncol, *9:* 247-256, 2008.

213. Kushner, B. H., LaQuaglia, M. P., Bonilla, M. A., Lindsley, K., Rosenfield, N., Yeh, S., Eddy, J., Gerald, W. L., Heller, G., and Cheung, N. K. Highly effective induction therapy for stage 4 neuroblastoma in children over 1 year of age. J Clin Oncol, *12:* 2607-2613, 1994.
214. Berthold, F., Boos, J., Burdach, S., Erttmann, R., Henze, G., Hermann, J., Klingebiel, T., Kremens, B., Schilling, F. H., Schrappe, M., Simon, T., and Hero, B. Myeloablative megatherapy with autologous stem-cell rescue versus oral maintenance chemotherapy as consolidation treatment in patients with high-risk neuroblastoma: a randomised controlled trial. Lancet Oncol, *6:* 649-658, 2005.
215. Knudson, A. G., Jr. and Strong, L. C. Mutation and cancer: neuroblastoma and pheochromocytoma. Am J Hum Genet, *24:* 514-532, 1972.
216. Knudson, A. G., Jr. and Meadows, A. T. Developmental genetics of neuroblastoma. J Natl Cancer Inst, *57:* 675-682, 1976.
217. Krause, A., Combaret, V., Iacono, I., Lacroix, B., Compagnon, C., Bergeron, C., Valsesia-Wittmann, S., Leissner, P., Mougin, B., and Puisieux, A. Genome-wide analysis of gene expression in neuroblastomas detected by mass screening. Cancer Lett, *225:* 111-120, 2005.
218. Schramm, A., Schulte, J. H., Klein-Hitpass, L., Havers, W., Sieverts, H., Berwanger, B., Christiansen, H., Warnat, P., Brors, B., Eils, J., Eils, R., and Eggert, A. Prediction of clinical outcome and biological characterization of neuroblastoma by expression profiling. Oncogene, *24:* 7902-7912, 2005.
219. Brodeur, G. M. and Fong, C. T. Molecular biology and genetics of human neuroblastoma. Cancer Genet Cytogenet, *41:* 153-174, 1989.
220. White, P. S., Maris, J. M., Beltinger, C., Sulman, E., Marshall, H. N., Fujimori, M., Kaufman, B. A., Biegel, J. A., Allen, C., Hilliard, C., Valentine, M. B., Look, A. T., Enomoto, H., Sakiyama, S., and Brodeur, G. M. A region of consistent deletion in neuroblastoma maps within human chromosome 1p36.2-36.3. Proc Natl Acad Sci U S A, *92:* 5520-5524, 1995.
221. Caron, H., van Sluis, P., de Kraker, J., Bokkerink, J., Egeler, M., Laureys, G., Slater, R., Westerveld, A., Voute, P. A., and Versteeg, R. Allelic loss of chromosome 1p as a predictor of unfavorable outcome in patients with neuroblastoma. N Engl J Med, *334:* 225-230, 1996.
222. Guo, C., White, P. S., Weiss, M. J., Hogarty, M. D., Thompson, P. M., Stram, D. O., Gerbing, R., Matthay, K. K., Seeger, R. C., Brodeur, G. M., and Maris, J. M. Allelic deletion at 11q23 is common in MYCN single copy neuroblastomas. Oncogene, *18:* 4948-4957, 1999.
223. Attiyeh, E. F., London, W. B., Mosse, Y. P., Wang, Q., Winter, C., Khazi, D., McGrady, P. W., Seeger, R. C., Look, A. T., Shimada, H., Brodeur, G. M., Cohn, S. L., Matthay, K. K., and Maris, J. M. Chromosome 1p and 11q deletions and outcome in neuroblastoma. N Engl J Med, *353:* 2243-2253, 2005.
224. Bown, N., Cotterill, S., Lastowska, M., O'Neill, S., Pearson, A. D., Plantaz, D., Meddeb, M., Danglot, G., Brinkschmidt, C., Christiansen, H., Laureys, G., Speleman, F., Nicholson, J., Bernheim, A., Betts, D. R., Vandesompele, J., and Van Roy, N. Gain of chromosome arm 17q and adverse outcome in patients with neuroblastoma. N Engl J Med, *340:* 1954-1961, 1999.
225. Lastowska, M., Cotterill, S., Pearson, A. D., Roberts, P., McGuckin, A., Lewis, I., and Bown, N. Gain of chromosome arm 17q predicts unfavourable outcome in neuroblastoma patients. U.K. Children's Cancer Study Group and the U.K. Cancer Cytogenetics Group. Eur J Cancer, *33:* 1627-1633, 1997.
226. Schleiermacher, G., Raynal, V., Janoueix-Lerosey, I., Combaret, V., Aurias, A., and Delattre, O. Variety and complexity of chromosome 17 translocations in neuroblastoma. Genes Chromosomes Cancer, *39:* 143-150, 2004.
227. , !!! INVALID CITATION !!!
228. Janoueix-Lerosey, I., Schleiermacher, G., Michels, E., Mosseri, V., Ribeiro, A., Lequin, D., Vermeulen, J., Couturier, J., Peuchmaur, M., Valent, A., Plantaz, D., Rubie, H., Valteau-Couanet, D., Thomas, C., Combaret, V., Rousseau, R., Eggert, A., Michon, J., Speleman, F., and Delattre, O. Overall genomic pattern is a predictor of outcome in neuroblastoma. J Clin Oncol, *27:* 1026-1033, 2009.
229. Mosse, Y. P., Laudenslager, M., Khazi, D., Carlisle, A. J., Winter, C. L., Rappaport, E., and Maris, J. M. Germline PHOX2B mutation in hereditary neuroblastoma. Am J Hum Genet, *75:* 727-730, 2004.
230. Trochet, D., Bourdeaut, F., Janoueix-Lerosey, I., Deville, A., de Pontual, L., Schleiermacher, G., Coze, C., Philip, N., Frebourg, T., Munnich, A., Lyonnet, S., Delattre, O., and Amiel, J. Germline mutations of the paired-like homeobox 2B (PHOX2B) gene in neuroblastoma. Am J Hum Genet, *74:* 761-764, 2004.
231. Capasso, M., Devoto, M., Hou, C., Asgharzadeh, S., Glessner, J. T., Attiyeh, E. F., Mosse, Y. P., Kim, C., Diskin, S. J., Cole, K. A., Bosse, K., Diamond, M., Laudenslager, M., Winter, C., Bradfield, J. P., Scott, R. H., Jagannathan, J., Garris, M., McConville, C., London, W. B., Seeger, R. C., Grant, S. F., Li, H., Rahman, N., Rappaport, E., Hakonarson, H., and Maris, J. M. Common variations in BARD1 influence susceptibility to high-risk neuroblastoma. Nat Genet, *41:* 718-723, 2009.
232. Maris, J. M., Mosse, Y. P., Bradfield, J. P., Hou, C., Monni, S., Scott, R. H., Asgharzadeh, S., Attiyeh, E. F., Diskin, S. J., Laudenslager, M., Winter, C., Cole, K. A., Glessner, J. T., Kim, C., Frackelton, E. C., Casalunovo, T., Eckert, A. W., Capasso, M., Rappaport, E. F., McConville, C., London, W. B., Seeger, R. C.,

Rahman, N., Devoto, M., Grant, S. F., Li, H., and Hakonarson, H. Chromosome 6p22 locus associated with clinically aggressive neuroblastoma. N Engl J Med, *358:* 2585-2593, 2008.

233. Mosse, Y. P., Laudenslager, M., Longo, L., Cole, K. A., Wood, A., Attiyeh, E. F., Laquaglia, M. J., Sennett, R., Lynch, J. E., Perri, P., Laureys, G., Speleman, F., Kim, C., Hou, C., Hakonarson, H., Torkamani, A., Schork, N. J., Brodeur, G. M., Tonini, G. P., Rappaport, E., Devoto, M., and Maris, J. M. Identification of ALK as a major familial neuroblastoma predisposition gene. Nature, *455:* 930-935, 2008.
234. Janoueix-Lerosey, I., Lequin, D., Brugieres, L., Ribeiro, A., de Pontual, L., Combaret, V., Raynal, V., Puisieux, A., Schleiermacher, G., Pierron, G., Valteau-Couanet, D., Frebourg, T., Michon, J., Lyonnet, S., Amiel, J., and Delattre, O. Somatic and germline activating mutations of the ALK kinase receptor in neuroblastoma. Nature, *455:* 967-970, 2008.
235. George, R. E., Sanda, T., Hanna, M., Frohling, S., Luther, W., 2nd, Zhang, J., Ahn, Y., Zhou, W., London, W. B., McGrady, P., Xue, L., Zozulya, S., Gregor, V. E., Webb, T. R., Gray, N. S., Gilliland, D. G., Diller, L., Greulich, H., Morris, S. W., Meyerson, M., and Look, A. T. Activating mutations in ALK provide a therapeutic target in neuroblastoma. Nature, *455:* 975-978, 2008.
236. Zhu, S., Lee, J. S., Guo, F., Shin, J., Perez-Atayde, A. R., Kutok, J. L., Rodig, S. J., Neuberg, D. S., Helman, D., Feng, H., Stewart, R. A., Wang, W., George, R. E., Kanki, J. P., and Look, A. T. Activated ALK collaborates with MYCN in neuroblastoma pathogenesis. Cancer Cell, *21:* 362-373, 2012.
237. Polkinghorn, W. R. and Tarbell, N. J. Medulloblastoma: tumorigenesis, current clinical paradigm, and efforts to improve risk stratification. Nat Clin Pract Oncol, *4:* 295-304, 2007.
238. McNeil, D. E., Cote, T. R., Clegg, L., and Rorke, L. B. Incidence and trends in pediatric malignancies medulloblastoma/primitive neuroectodermal tumor: a SEER update. Surveillance Epidemiology and End Results. Med Pediatr Oncol, *39:* 190-194, 2002.
239. Legler, J. M., Gloeckler Ries, L. A., Smith, M. A., Warren, J. L., Heineman, E. F., Kaplan, R. S., and Linet, M. S. RESPONSE: Re: Brain and Other Central Nervous System Cancers: Recent Trends in Incidence and Mortality. J Natl Cancer Inst, *91:* 2050A-22051, 1999.
240. Crawford, J. R., MacDonald, T. J., and Packer, R. J. Medulloblastoma in childhood: new biological advances. Lancet Neurol, *6:* 1073-1085, 2007.
241. Chan, A. W., Tarbell, N. J., Black, P. M., Louis, D. N., Frosch, M. P., Ancukiewicz, M., Chapman, P., and Loeffler, J. S. Adult medulloblastoma: prognostic factors and patterns of relapse. Neurosurgery, *47:* 623-631; discussion 631-622, 2000.
242. Engelhard, H. H. and Corsten, L. A. Leptomeningeal metastasis of primary central nervous system (CNS) neoplasms. Cancer Treat Res, *125:* 71-85, 2005.
243. Allen, J. C. and Epstein, F. Medulloblastoma and other primary malignant neuroectodermal tumors of the CNS. The effect of patients' age and extent of disease on prognosis. J Neurosurg, *57:* 446-451, 1982.
244. Parsons, D. W., Li, M., Zhang, X., Jones, S., Leary, R. J., Lin, J. C., Boca, S. M., Carter, H., Samayoa, J., Bettegowda, C., Gallia, G. L., Jallo, G. I., Binder, Z. A., Nikolsky, Y., Hartigan, J., Smith, D. R., Gerhard, D. S., Fults, D. W., VandenBerg, S., Berger, M. S., Marie, S. K., Shinjo, S. M., Clara, C., Phillips, P. C., Minturn, J. E., Biegel, J. A., Judkins, A. R., Resnick, A. C., Storm, P. B., Curran, T., He, Y., Rasheed, B. A., Friedman, H. S., Keir, S. T., McLendon, R., Northcott, P. A., Taylor, M. D., Burger, P. C., Riggins, G. J., Karchin, R., Parmigiani, G., Bigner, D. D., Yan, H., Papadopoulos, N., Vogelstein, B., Kinzler, K. W., and Velculescu, V. E. The genetic landscape of the childhood cancer medulloblastoma. Science, *331:* 435-439, 2011.
245. Bigner, S. H., Mark, J., Friedman, H. S., Biegel, J. A., and Bigner, D. D. Structural chromosomal abnormalities in human medulloblastoma. Cancer Genet Cytogenet, *30:* 91-101, 1988.
246. Gilbertson, R. J. and Ellison, D. W. The origins of medulloblastoma subtypes. Annu Rev Pathol, *3:* 341-365, 2008.
247. Lamont, J. M., McManamy, C. S., Pearson, A. D., Clifford, S. C., and Ellison, D. W. Combined histopathological and molecular cytogenetic stratification of medulloblastoma patients. Clin Cancer Res, *10:* 5482-5493, 2004.
248. Aldosari, N., Bigner, S. H., Burger, P. C., Becker, L., Kepner, J. L., Friedman, H. S., and McLendon, R. E. MYCC and MYCN oncogene amplification in medulloblastoma. A fluorescence in situ hybridization study on paraffin sections from the Children's Oncology Group. Arch Pathol Lab Med, *126:* 540-544, 2002.
249. Reardon, D. A., Michalkiewicz, E., Boyett, J. M., Sublett, J. E., Entrekin, R. E., Ragsdale, S. T., Valentine, M. B., Behm, F. G., Li, H., Heideman, R. L., Kun, L. E., Shapiro, D. N., and Look, A. T. Extensive genomic abnormalities in childhood medulloblastoma by comparative genomic hybridization. Cancer Res, *57:* 4042-4047, 1997.
250. Clifford, S. C., Lusher, M. E., Lindsey, J. C., Langdon, J. A., Gilbertson, R. J., Straughton, D., and Ellison, D. W. Wnt/Wingless pathway activation and chromosome 6 loss characterize a distinct molecular sub-group of medulloblastomas associated with a favorable prognosis. Cell Cycle, *5:* 2666-2670, 2006.

251. Eberhart, C. G., Kepner, J. L., Goldthwaite, P. T., Kun, L. E., Duffner, P. K., Friedman, H. S., Strother, D. R., and Burger, P. C. Histopathologic grading of medulloblastomas: a Pediatric Oncology Group study. Cancer, *94:* 552-560, 2002.
252. Pomeroy, S. L., Tamayo, P., Gaasenbeek, M., Sturla, L. M., Angelo, M., McLaughlin, M. E., Kim, J. Y., Goumnerova, L. C., Black, P. M., Lau, C., Allen, J. C., Zagzag, D., Olson, J. M., Curran, T., Wetmore, C., Biegel, J. A., Poggio, T., Mukherjee, S., Rifkin, R., Califano, A., Stolovitzky, G., Louis, D. N., Mesirov, J. P., Lander, E. S., and Golub, T. R. Prediction of central nervous system embryonal tumour outcome based on gene expression. Nature, *415:* 436-442, 2002.
253. Taylor, M. D., Northcott, P. A., Korshunov, A., Remke, M., Cho, Y. J., Clifford, S. C., Eberhart, C. G., Parsons, D. W., Rutkowski, S., Gajjar, A., Ellison, D. W., Lichter, P., Gilbertson, R. J., Pomeroy, S. L., Kool, M., and Pfister, S. M. Molecular subgroups of medulloblastoma: the current consensus. Acta Neuropathol, *123:* 465-472, 2012.
254. Kool, M., Korshunov, A., Remke, M., Jones, D. T., Schlanstein, M., Northcott, P. A., Cho, Y. J., Koster, J., Schouten-van Meeteren, A., van Vuurden, D., Clifford, S. C., Pietsch, T., von Bueren, A. O., Rutkowski, S., McCabe, M., Collins, V. P., Backlund, M. L., Haberler, C., Bourdeaut, F., Delattre, O., Doz, F., Ellison, D. W., Gilbertson, R. J., Pomeroy, S. L., Taylor, M. D., Lichter, P., and Pfister, S. M. Molecular subgroups of medulloblastoma: an international meta-analysis of transcriptome, genetic aberrations, and clinical data of WNT, SHH, Group 3, and Group 4 medulloblastomas. Acta Neuropathol, *123:* 473-484, 2012.
255. De Meyts, P., Wallach, B., Christoffersen, C. T., Urso, B., Gronskov, K., Latus, L. J., Yakushiji, F., Ilondo, M. M., and Shymko, R. M. The insulin-like growth factor-I receptor. Structure, ligand-binding mechanism and signal transduction. Horm Res, *42:* 152-169, 1994.
256. Jones, J. I. and Clemmons, D. R. Insulin-like growth factors and their binding proteins: biological actions. Endocr Rev, *16:* 3-34, 1995.
257. Lee, J. and Pilch, P. F. The insulin receptor: structure, function, and signaling. Am J Physiol, *266:* C319-334, 1994.
258. Blakesley, V. A., Scrimgeour, A., Esposito, D., and Le Roith, D. Signaling via the insulin-like growth factor-I receptor: does it differ from insulin receptor signaling? Cytokine Growth Factor Rev, *7:* 153-159, 1996.
259. Patti, M. E. and Kahn, C. R. The insulin receptor--a critical link in glucose homeostasis and insulin action. J Basic Clin Physiol Pharmacol, *9:* 89-109, 1998.
260. Urso, B., Cope, D. L., Kalloo-Hosein, H. E., Hayward, A. C., Whitehead, J. P., O'Rahilly, S., and Siddle, K. Differences in signaling properties of the cytoplasmic domains of the insulin receptor and insulin-like growth factor receptor in 3T3-L1 adipocytes. J Biol Chem, *274:* 30864-30873, 1999.
261. Ullrich, A., Gray, A., Tam, A. W., Yang-Feng, T., Tsubokawa, M., Collins, C., Henzel, W., Le Bon, T., Kathuria, S., Chen, E., and et al. Insulin-like growth factor I receptor primary structure: comparison with insulin receptor suggests structural determinants that define functional specificity. EMBO J, *5:* 2503-2512, 1986.
262. Denley, A., Bonython, E. R., Booker, G. W., Cosgrove, L. J., Forbes, B. E., Ward, C. W., and Wallace, J. C. Structural determinants for high-affinity binding of insulin-like growth factor II to insulin receptor (IR)-A, the exon 11 minus isoform of the IR. Mol Endocrinol, *18:* 2502-2512, 2004.
263. Baserga, R., Hongo, A., Rubini, M., Prisco, M., and Valentinis, B. The IGF-I receptor in cell growth, transformation and apoptosis. Biochim Biophys Acta, *1332:* F105-126, 1997.
264. Pollak, M. N., Schernhammer, E. S., and Hankinson, S. E. Insulin-like growth factors and neoplasia. Nat Rev Cancer, *4:* 505-518, 2004.
265. Peruzzi, F., Prisco, M., Dews, M., Salomoni, P., Grassilli, E., Romano, G., Calabretta, B., and Baserga, R. Multiple signaling pathways of the insulin-like growth factor 1 receptor in protection from apoptosis. Mol Cell Biol, *19:* 7203-7215, 1999.
266. Liu, J. P., Baker, J., Perkins, A. S., Robertson, E. J., and Efstratiadis, A. Mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 IGF receptor (Igf1r). Cell, *75:* 59-72, 1993.
267. Shevah, O. and Laron, Z. Patients with congenital deficiency of IGF-I seem protected from the development of malignancies: a preliminary report. Growth Horm IGF Res, *17:* 54-57, 2007.
268. Meyer, G. E., Shelden, E., Kim, B., and Feldman, E. L. Insulin-like growth factor I stimulates motility in human neuroblastoma cells. Oncogene, *20:* 7542-7550, 2001.
269. Singleton, J. R., Randolph, A. E., and Feldman, E. L. Insulin-like growth factor I receptor prevents apoptosis and enhances neuroblastoma tumorigenesis. Cancer Res, *56:* 4522-4529, 1996.
270. El-Badry, O. M., Helman, L. J., Chatten, J., Steinberg, S. M., Evans, A. E., and Israel, M. A. Insulin-like growth factor II-mediated proliferation of human neuroblastoma. J Clin Invest, *87:* 648-657, 1991.
271. van Golen, C. M., Schwab, T. S., Kim, B., Soules, M. E., Su Oh, S., Fung, K., van Golen, K. L., and Feldman, E. L. Insulin-like growth factor-I receptor expression regulates neuroblastoma metastasis to bone. Cancer Res, *66:* 6570-6578, 2006.

272. Cianfarani, S. and Rossi, P. Neuroblastoma and insulin-like growth factor system. New insights and clinical perspectives. Eur J Pediatr, *156:* 256-261, 1997.
273. Izycka-Swieszewska, E., Brzeskwiniewicz, M., Wozniak, A., Drozynska, E., Grajkowska, W., Perek, D., Balcerska, A., Klepacka, T., and Limon, J. EGFR, PIK3CA and PTEN gene status and their protein product expression in neuroblastic tumours. Folia Neuropathol, *48:* 238-245, 2010.
274. Dam, V., Morgan, B. T., Mazanek, P., and Hogarty, M. D. Mutations in PIK3CA are infrequent in neuroblastoma. BMC Cancer, *6:* 177, 2006.
275. Mosse, Y. P., Greshock, J., Margolin, A., Naylor, T., Cole, K., Khazi, D., Hii, G., Winter, C., Shahzad, S., Asziz, M. U., Biegel, J. A., Weber, B. L., and Maris, J. M. High-resolution detection and mapping of genomic DNA alterations in neuroblastoma. Genes Chromosomes Cancer, *43:* 390-403, 2005.
276. Caren, H., Fransson, S., Ejeskar, K., Kogner, P., and Martinsson, T. Genetic and epigenetic changes in the common 1p36 deletion in neuroblastoma tumours. Br J Cancer, *97:* 1416-1424, 2007.
277. Boller, D., Schramm, A., Doepfner, K. T., Shalaby, T., von Bueren, A. O., Eggert, A., Grotzer, M. A., and Arcaro, A. Targeting the phosphoinositide 3-kinase isoform p110delta impairs growth and survival in neuroblastoma cells. Clin Cancer Res, *14:* 1172-1181, 2008.
278. Moritake, H., Horii, Y., Kuroda, H., and Sugimoto, T. Analysis of PTEN/MMAC1 alteration in neuroblastoma. Cancer Genet Cytogenet, *125:* 151-155, 2001.
279. Lazcoz, P., Munoz, J., Nistal, M., Pestana, A., Encio, I. J., and Castresana, J. S. Loss of heterozygosity and microsatellite instability on chromosome arm 10q in neuroblastoma. Cancer Genet Cytogenet, *174:* 1-8, 2007.
280. Opel, D., Poremba, C., Simon, T., Debatin, K. M., and Fulda, S. Activation of Akt predicts poor outcome in neuroblastoma. Cancer Res, *67:* 735-745, 2007.
281. Del Valle, L., Enam, S., Lassak, A., Wang, J. Y., Croul, S., Khalili, K., and Reiss, K. Insulin-like growth factor I receptor activity in human medulloblastomas. Clin Cancer Res, *8:* 1822-1830, 2002.
282. Grotzer, M. A., Janss, A. J., Fung, K., Biegel, J. A., Sutton, L. N., Rorke, L. B., Zhao, H., Cnaan, A., Phillips, P. C., Lee, V. M., and Trojanowski, J. Q. TrkC expression predicts good clinical outcome in primitive neuroectodermal brain tumors. J Clin Oncol, *18:* 1027-1035, 2000.
283. Broderick, D. K., Di, C., Parrett, T. J., Samuels, Y. R., Cummins, J. M., McLendon, R. E., Fults, D. W., Velculescu, V. E., Bigner, D. D., and Yan, H. Mutations of PIK3CA in anaplastic oligodendrogliomas, high-grade astrocytomas, and medulloblastomas. Cancer Res, *64:* 5048-5050, 2004.
284. Guerreiro, A. S., Fattet, S., Fischer, B., Shalaby, T., Jackson, S. P., Schoenwaelder, S. M., Grotzer, M. A., Delattre, O., and Arcaro, A. Targeting the PI3K p110alpha isoform inhibits medulloblastoma proliferation, chemoresistance, and migration. Clin Cancer Res, *14:* 6761-6769, 2008.
285. Guerreiro, A. S., Fattet, S., Kulesza, D. W., Atamer, A., Elsing, A. N., Shalaby, T., Jackson, S. P., Schoenwaelder, S. M., Grotzer, M. A., Delattre, O., and Arcaro, A. A sensitized RNA interference screen identifies a novel role for the PI3K p110gamma isoform in medulloblastoma cell proliferation and chemoresistance. Mol Cancer Res, *9:* 925-935, 2011.
286. Hartmann, W., Digon-Sontgerath, B., Koch, A., Waha, A., Endl, E., Dani, I., Denkhaus, D., Goodyer, C. G., Sorensen, N., Wiestler, O. D., and Pietsch, T. Phosphatidylinositol 3'-kinase/AKT signaling is activated in medulloblastoma cell proliferation and is associated with reduced expression of PTEN. Clin Cancer Res, *12:* 3019-3027, 2006.
287. Inda, M. M., Mercapide, J., Munoz, J., Coullin, P., Danglot, G., Tunon, T., Martinez-Penuela, J. M., Rivera, J. M., Burgos, J. J., Bernheim, A., and Castresana, J. S. PTEN and DMBT1 homozygous deletion and expression in medulloblastomas and supratentorial primitive neuroectodermal tumors. Oncol Rep, *12:* 1341-1347, 2004.
288. Opel, D., Naumann, I., Schneider, M., Bertele, D., Debatin, K. M., and Fulda, S. Targeting aberrant PI3K/Akt activation by PI103 restores sensitivity to TRAIL-induced apoptosis in neuroblastoma. Clin Cancer Res, *17:* 3233-3247, 2011.
289. Segerstrom, L., Baryawno, N., Sveinbjornsson, B., Wickstrom, M., Elfman, L., Kogner, P., and Johnsen, J. I. Effects of small molecule inhibitors of PI3K/Akt/mTOR signaling on neuroblastoma growth in vitro and in vivo. Int J Cancer, *129:* 2958-2965, 2011.
290. Baryawno, N., Sveinbjornsson, B., Eksborg, S., Chen, C. S., Kogner, P., and Johnsen, J. I. Small-molecule inhibitors of phosphatidylinositol 3-kinase/Akt signaling inhibit Wnt/beta-catenin pathway cross-talk and suppress medulloblastoma growth. Cancer Res, *70:* 266-276, 2010.
291. Kumar, C. C. Signaling by integrin receptors. Oncogene, *17:* 1365-1373, 1998.
292. Garcia-Echeverria, C. and Sellers, W. R. Drug discovery approaches targeting the PI3K/Akt pathway in cancer. Oncogene, *27:* 5511-5526, 2008.
293. Wojtalla, A. and Arcaro, A. Targeting phosphoinositide 3-kinase signalling in lung cancer. Crit Rev Oncol Hematol, *80:* 278-290, 2011.

294. Shi, S. R., Key, M. E., and Kalra, K. L. Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. J Histochem Cytochem, *39:* 741-748, 1991.
295. Tschan, M. P., Fischer, K. M., Fung, V. S., Pirnia, F., Borner, M. M., Fey, M. F., Tobler, A., and Torbett, B. E. Alternative splicing of the human cyclin D-binding Myb-like protein (hDMP1) yields a truncated protein isoform that alters macrophage differentiation patterns. J Biol Chem, *278:* 42750-42760, 2003.
296. Brown, K. C., Witte, T. R., Hardman, W. E., Luo, H., Chen, Y. C., Carpenter, A. B., Lau, J. K., and Dasgupta, P. Capsaicin displays anti-proliferative activity against human small cell lung cancer in cell culture and nude mice models via the E2F pathway. PLoS One, *5:* e10243, 2010.
297. Sofka, M. and Stewart, C. V. Retinal vessel centerline extraction using multiscale matched filters, confidence and edge measures. IEEE Trans Med Imaging, *25:* 1531-1546, 2006.
298. Irizarry, R. A., Bolstad, B. M., Collin, F., Cope, L. M., Hobbs, B., and Speed, T. P. Summaries of Affymetrix GeneChip probe level data. Nucleic Acids Res, *31:* e15, 2003.
299. Chaussade, C., Rewcastle, G. W., Kendall, J. D., Denny, W. A., Cho, K., Gronning, L. M., Chong, M. L., Anagnostou, S. H., Jackson, S. P., Daniele, N., and Shepherd, P. R. Evidence for functional redundancy of class IA PI3K isoforms in insulin signalling. Biochem J, *404:* 449-458, 2007.
300. Seckl, M. J., Higgins, T., Widmer, F., and Rozengurt, E. [D-Arg1,D-Trp5,7,9,Leu11]substance P: a novel potent inhibitor of signal transduction and growth in vitro and in vivo in small cell lung cancer cells. Cancer Res, *57:* 51-54, 1997.
301. Turco, M. C., Romano, M. F., Petrella, A., Bisogni, R., Tassone, P., and Venuta, S. NF-kappaB/Rel-mediated regulation of apoptosis in hematologic malignancies and normal hematopoietic progenitors. Leukemia, *18:* 11-17, 2004.
302. Brambilla, E., Negoescu, A., Gazzeri, S., Lantuejoul, S., Moro, D., Brambilla, C., and Coll, J. L. Apoptosis-related factors p53, Bcl2, and Bax in neuroendocrine lung tumors. Am J Pathol, *149:* 1941-1952, 1996.
303. Han, J. Y., Chung, Y. J., Park, S. W., Kim, J. S., Rhyu, M. G., Kim, H. K., and Lee, K. S. The relationship between cisplatin-induced apoptosis and p53, bcl-2 and bax expression in human lung cancer cells. Korean J Intern Med, *14:* 42-52, 1999.
304. Marinov, M., Ziogas, A., Pardo, O. E., Tan, L. T., Dhillon, T., Mauri, F. A., Lane, H. A., Lemoine, N. R., Zangemeister-Wittke, U., Seckl, M. J., and Arcaro, A. AKT/mTOR pathway activation and BCL-2 family proteins modulate the sensitivity of human small cell lung cancer cells to RAD001. Clin Cancer Res, *15:* 1277-1287, 2009.
305. Santos, A. O., Pereira, J. P., Pedroso de Lima, M. C., Simoes, S., and Moreira, J. N. In vitro modulation of Bcl-2 levels in small cell lung cancer cells: effects on cell viability. Braz J Med Biol Res, *43:* 1001-1009.
306. Sasi, N., Hwang, M., Jaboin, J., Csiki, I., and Lu, B. Regulated cell death pathways: new twists in modulation of BCL2 family function. Mol Cancer Ther, *8:* 1421-1429, 2009.
307. Foukas, L. C., Berenjeno, I. M., Gray, A., Khwaja, A., and Vanhaesebroeck, B. Activity of any class IA PI3K isoform can sustain cell proliferation and survival. Proc Natl Acad Sci U S A, *107:* 11381-11386, 2010.
308. Schwab, M., Praml, C., and Amler, L. C. Genomic instability in 1p and human malignancies. Genes Chromosomes Cancer, *16:* 211-229, 1996.
309. Schwab, M., Alitalo, K., Klempnauer, K. H., Varmus, H. E., Bishop, J. M., Gilbert, F., Brodeur, G., Goldstein, M., and Trent, J. Amplified DNA with limited homology to myc cellular oncogene is shared by human neuroblastoma cell lines and a neuroblastoma tumour. Nature, *305:* 245-248, 1983.
310. Plantaz, D., Vandesompele, J., Van Roy, N., Lastowska, M., Bown, N., Combaret, V., Favrot, M. C., Delattre, O., Michon, J., Benard, J., Hartmann, O., Nicholson, J. C., Ross, F. M., Brinkschmidt, C., Laureys, G., Caron, H., Matthay, K. K., Feuerstein, B. G., and Speleman, F. Comparative genomic hybridization (CGH) analysis of stage 4 neuroblastoma reveals high frequency of 11q deletion in tumors lacking MYCN amplification. Int J Cancer, *91:* 680-686, 2001.
311. Van Roy, N., Van Limbergen, H., Vandesompele, J., Van Gele, M., Poppe, B., Salwen, H., Laureys, G., Manoel, N., De Paepe, A., and Speleman, F. Combined M-FISH and CGH analysis allows comprehensive description of genetic alterations in neuroblastoma cell lines. Genes Chromosomes Cancer, *32:* 126-135, 2001.
312. Van Roy, N., Jauch, A., Van Gele, M., Laureys, G., Versteeg, R., De Paepe, A., Cremer, T., and Speleman, F. Comparative genomic hybridization analysis of human neuroblastomas: detection of distal 1p deletions and further molecular genetic characterization of neuroblastoma cell lines. Cancer Genet Cytogenet, *97:* 135-142, 1997.
313. Van Roy, N., Cheng, N. C., Laureys, G., Opdenakker, G., Versteeg, R., and Speleman, F. Molecular cytogenetic analysis of 1;17 translocations in neuroblastoma. Eur J Cancer, *31A:* 530-535, 1995.

314. Delattre, O., Zucman, J., Plougastel, B., Desmaze, C., Melot, T., Peter, M., Kovar, H., Joubert, I., de Jong, P., Rouleau, G., and et al. Gene fusion with an ETS DNA-binding domain caused by chromosome translocation in human tumours. Nature, *359:* 162-165, 1992.
315. Kovar, H., Aryee, D. N., Jug, G., Henockl, C., Schemper, M., Delattre, O., Thomas, G., and Gadner, H. EWS/FLI-1 antagonists induce growth inhibition of Ewing tumor cells in vitro. Cell Growth Differ, *7:* 429-437, 1996.
316. Zoubek, A., Dockhorn-Dworniczak, B., Delattre, O., Christiansen, H., Niggli, F., Gatterer-Menz, I., Smith, T. L., Jurgens, H., Gadner, H., and Kovar, H. Does expression of different EWS chimeric transcripts define clinically distinct risk groups of Ewing tumor patients? J Clin Oncol, *14:* 1245-1251, 1996.
317. Grotzer, M. A., Castelletti, D., Fiaschetti, G., Shalaby, T., and Arcaro, A. Targeting Myc in pediatric malignancies of the central and peripheral nervous system. Curr Cancer Drug Targets, *9:* 176-188, 2009.
318. Wechsler-Reya, R. and Scott, M. P. The developmental biology of brain tumors. Annu Rev Neurosci, *24:* 385-428, 2001.
319. Gilbertson, R. Paediatric embryonic brain tumours. biological and clinical relevance of molecular genetic abnormalities. Eur J Cancer, *38:* 675-685, 2002.
320. Ho, R., Eggert, A., Hishiki, T., Minturn, J. E., Ikegaki, N., Foster, P., Camoratto, A. M., Evans, A. E., and Brodeur, G. M. Resistance to chemotherapy mediated by TrkB in neuroblastomas. Cancer Res, *62:* 6462-6466, 2002.
321. Rikhof, B., de Jong, S., Suurmeijer, A. J., Meijer, C., and van der Graaf, W. T. The insulin-like growth factor system and sarcomas. J Pathol, *217:* 469-482, 2009.
322. Kim, S. Y., Wan, X., and Helman, L. J. Targeting IGF-1R in the treatment of sarcomas: past, present and future. Bull Cancer, *96:* E52-60, 2009.
323. Kim, S. Y., Toretsky, J. A., Scher, D., and Helman, L. J. The role of IGF-1R in pediatric malignancies. Oncologist, *14:* 83-91, 2009.
324. Tanno, B., Mancini, C., Vitali, R., Mancuso, M., McDowell, H. P., Dominici, C., and Raschella, G. Down-regulation of insulin-like growth factor I receptor activity by NVP-AEW541 has an antitumor effect on neuroblastoma cells in vitro and in vivo. Clin Cancer Res, *12:* 6772-6780, 2006.
325. Manara, M. C., Landuzzi, L., Nanni, P., Nicoletti, G., Zambelli, D., Lollini, P. L., Nanni, C., Hofmann, F., Garcia-Echeverria, C., Picci, P., and Scotlandi, K. Preclinical in vivo study of new insulin-like growth factor-I receptor--specific inhibitor in Ewing's sarcoma. Clin Cancer Res, *13:* 1322-1330, 2007.
326. Guerreiro, A. S., Boller, D., Shalaby, T., Grotzer, M. A., and Arcaro, A. Protein kinase B modulates the sensitivity of human neuroblastoma cells to insulin-like growth factor receptor inhibition. Int J Cancer, *119:* 2527-2538, 2006.
327. Martins, A. S., Mackintosh, C., Martin, D. H., Campos, M., Hernandez, T., Ordonez, J. L., and de Alava, E. Insulin-like growth factor I receptor pathway inhibition by ADW742, alone or in combination with imatinib, doxorubicin, or vincristine, is a novel therapeutic approach in Ewing tumor. Clin Cancer Res, *12:* 3532-3540, 2006.
328. Pollak, M. Targeting insulin and insulin-like growth factor signalling in oncology. Curr Opin Pharmacol, *8:* 384-392, 2008.
329. Pollak, M. Insulin and insulin-like growth factor signalling in neoplasia. Nat Rev Cancer, *8:* 915-928, 2008.
330. Kurzrock, R., Patnaik, A., Aisner, J., Warren, T., Leong, S., Benjamin, R., Eckhardt, S. G., Eid, J. E., Greig, G., Habben, K., McCarthy, C. D., and Gore, L. A phase I study of weekly R1507, a human monoclonal antibody insulin-like growth factor-I receptor antagonist, in patients with advanced solid tumors. Clin Cancer Res, *16:* 2458-2465, 2010.
331. Pappo, A. S., Patel, S. R., Crowley, J., Reinke, D. K., Kuenkele, K. P., Chawla, S. P., Toner, G. C., Maki, R. G., Meyers, P. A., Chugh, R., Ganjoo, K. N., Schuetze, S. M., Juergens, H., Leahy, M. G., Geoerger, B., Benjamin, R. S., Helman, L. J., and Baker, L. H. R1507, a monoclonal antibody to the insulin-like growth factor 1 receptor, in patients with recurrent or refractory Ewing sarcoma family of tumors: results of a phase II Sarcoma Alliance for Research through Collaboration study. J Clin Oncol, *29:* 4541-4547, 2011.
332. Yee, D., Favoni, R. E., Lebovic, G. S., Lombana, F., Powell, D. R., Reynolds, C. P., and Rosen, N. Insulin-like growth factor I expression by tumors of neuroectodermal origin with the t(11;22) chromosomal translocation. A potential autocrine growth factor. J Clin Invest, *86:* 1806-1814, 1990.
333. Kuwahara, Y., Hosoi, H., Osone, S., Kita, M., Iehara, T., Kuroda, H., and Sugimoto, T. Antitumor activity of gefitinib in malignant rhabdoid tumor cells in vitro and in vivo. Clin Cancer Res, *10:* 5940-5948, 2004.
334. Wewetzer, K., Janet, T., Heymann, D., and Unsicker, K. Cell blotting and isoelectric focusing of neuroblastoma-derived heparin-binding neurotrophic activities: detection of basic fibroblast growth factor protein and mRNA. J Neurosci Res, *36:* 209-215, 1993.
335. Cohen, P. S., Chan, J. P., Lipkunskaya, M., Biedler, J. L., and Seeger, R. C. Expression of stem cell factor and c-kit in human neuroblastoma. The Children's Cancer Group. Blood, *84:* 3465-3472, 1994.

336. Gilbertson, R. J., Perry, R. H., Kelly, P. J., Pearson, A. D., and Lunec, J. Prognostic significance of HER2 and HER4 coexpression in childhood medulloblastoma. Cancer Res, *57:* 3272-3280, 1997.
337. Gilbertson, R. J., Clifford, S. C., MacMeekin, W., Meekin, W., Wright, C., Perry, R. H., Kelly, P., Pearson, A. D., and Lunec, J. Expression of the ErbB-neuregulin signaling network during human cerebellar development: implications for the biology of medulloblastoma. Cancer Res, *58:* 3932-3941, 1998.
338. MacDonald, T. J., Brown, K. M., LaFleur, B., Peterson, K., Lawlor, C., Chen, Y., Packer, R. J., Cogen, P., and Stephan, D. A. Expression profiling of medulloblastoma: PDGFRA and the RAS/MAPK pathway as therapeutic targets for metastatic disease. Nat Genet, *29:* 143-152, 2001.
339. Andrae, J., Molander, C., Smits, A., Funa, K., and Nister, M. Platelet-derived growth factor-B and -C and active alpha-receptors in medulloblastoma cells. Biochem Biophys Res Commun, *296:* 604-611, 2002.
340. Wang, J. Y., Del Valle, L., Gordon, J., Rubini, M., Romano, G., Croul, S., Peruzzi, F., Khalili, K., and Reiss, K. Activation of the IGF-IR system contributes to malignant growth of human and mouse medulloblastomas. Oncogene, *20:* 3857-3868, 2001.
341. Nakagawara, A. Trk receptor tyrosine kinases: a bridge between cancer and neural development. Cancer Lett, *169:* 107-114, 2001.
342. Duplan, S. M., Theoret, Y., and Kenigsberg, R. L. Antitumor activity of fibroblast growth factors (FGFs) for medulloblastoma may correlate with FGF receptor expression and tumor variant. Clin Cancer Res, *8:* 246-257, 2002.
343. Beppu, K., Jaboine, J., Merchant, M. S., Mackall, C. L., and Thiele, C. J. Effect of imatinib mesylate on neuroblastoma tumorigenesis and vascular endothelial growth factor expression. J Natl Cancer Inst, *96:* 46-55, 2004.
344. D'Cunja, J., Shalaby, T., Rivera, P., von Buren, A., Patti, R., Heppner, F. L., Arcaro, A., Rorke-Adams, L. B., Phillips, P. C., and Grotzer, M. A. Antisense treatment of IGF-IR induces apoptosis and enhances chemosensitivity in central nervous system atypical teratoid/rhabdoid tumours cells. Eur J Cancer, *43:* 1581-1589, 2007.
345. Arcaro, A., Doepfner, K. T., Boller, D., Guerreiro, A. S., Shalaby, T., Jackson, S. P., Schoenwaelder, S. M., Delattre, O., Grotzer, M. A., and Fischer, B. Novel role for insulin as an autocrine growth factor for malignant brain tumour cells. Biochem J, *406:* 57-66, 2007.
346. Marinov, M., Fischer, B., and Arcaro, A. Targeting mTOR signaling in lung cancer. Crit Rev Oncol Hematol, *63:* 172-182, 2007.
347. Kolb, E. A., Kamara, D., Zhang, W., Lin, J., Hingorani, P., Baker, L., Houghton, P., and Gorlick, R. R1507, a fully human monoclonal antibody targeting IGF-1R, is effective alone and in combination with rapamycin in inhibiting growth of osteosarcoma xenografts. Pediatr Blood Cancer, *55:* 67-75, 2010.
348. Wojtalla, A. and Arcaro, A. Targeting phosphoinositide 3-kinase signalling in lung cancer. Crit Rev Oncol Hematol, 2011.
349. Vanhaesebroeck, B., Vogt, P. K., and Rommel, C. PI3K: from the bench to the clinic and back. Curr Top Microbiol Immunol, *347:* 1-19, 2010.
350. Vanhaesebroeck, B., Guillermet-Guibert, J., Graupera, M., and Bilanges, B. The emerging mechanisms of isoform-specific PI3K signalling. Nat Rev Mol Cell Biol, *11:* 329-341, 2010.
351. Blajecka, K., Borgstrom, A., and Arcaro, A. Phosphatidylinositol 3-kinase isoforms as novel drug targets. Curr Drug Targets, *12:* 1056-1081, 2011.
352. Cantley, L. C. and Neel, B. G. New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway. Proc Natl Acad Sci U S A, *96:* 4240-4245, 1999.
353. Sansal, I. and Sellers, W. R. The biology and clinical relevance of the PTEN tumor suppressor pathway. J Clin Oncol, *22:* 2954-2963, 2004.
354. Arcaro, A. and Guerreiro, A. S. The phosphoinositide 3-kinase pathway in human cancer: genetic alterations and therapeutic implications. Curr Genomics, *8:* 271-306, 2007.
355. Hoebeeck, J., Michels, E., Pattyn, F., Combaret, V., Vermeulen, J., Yigit, N., Hoyoux, C., Laureys, G., De Paepe, A., Speleman, F., and Vandesompele, J. Aberrant methylation of candidate tumor suppressor genes in neuroblastoma. Cancer Lett, *273:* 336-346, 2009.
356. Samuels, Y., Wang, Z., Bardelli, A., Silliman, N., Ptak, J., Szabo, S., Yan, H., Gazdar, A., Powell, S. M., Riggins, G. J., Willson, J. K., Markowitz, S., Kinzler, K. W., Vogelstein, B., and Velculescu, V. E. High frequency of mutations of the PIK3CA gene in human cancers. Science, *304:* 554, 2004.
357. Kenney, A. M., Widlund, H. R., and Rowitch, D. H. Hedgehog and PI-3 kinase signaling converge on Nmyc1 to promote cell cycle progression in cerebellar neuronal precursors. Development, *131:* 217-228, 2004.
358. Chesler, L., Schlieve, C., Goldenberg, D. D., Kenney, A., Kim, G., McMillan, A., Matthay, K. K., Rowitch, D., and Weiss, W. A. Inhibition of phosphatidylinositol 3-kinase destabilizes Mycn protein and blocks malignant progression in neuroblastoma. Cancer Res, *66:* 8139-8146, 2006.

359. Hernan, R., Fasheh, R., Calabrese, C., Frank, A. J., Maclean, K. H., Allard, D., Barraclough, R., and Gilbertson, R. J. ERBB2 up-regulates S100A4 and several other prometastatic genes in medulloblastoma. Cancer Res, *63:* 140-148, 2003.
360. Kim, B., van Golen, C. M., and Feldman, E. L. Insulin-like growth factor-I signaling in human neuroblastoma cells. Oncogene, *23:* 130-141, 2004.
361. Sartelet, H., Oligny, L. L., and Vassal, G. AKT pathway in neuroblastoma and its therapeutic implication. Expert Rev Anticancer Ther, *8:* 757-769, 2008.
362. Yap, T. A., Garrett, M. D., Walton, M. I., Raynaud, F., de Bono, J. S., and Workman, P. Targeting the PI3K-AKT-mTOR pathway: progress, pitfalls, and promises. Curr Opin Pharmacol, *8:* 393-412, 2008.
363. Brachmann, S., Fritsch, C., Maira, S. M., and Garcia-Echeverria, C. PI3K and mTOR inhibitors: a new generation of targeted anticancer agents. Curr Opin Cell Biol, *21:* 194-198, 2009.
364. Sequist, L. V., Waltman, B. A., Dias-Santagata, D., Digumarthy, S., Turke, A. B., Fidias, P., Bergethon, K., Shaw, A. T., Gettinger, S., Cosper, A. K., Akhavanfard, S., Heist, R. S., Temel, J., Christensen, J. G., Wain, J. C., Lynch, T. J., Vernovsky, K., Mark, E. J., Lanuti, M., Iafrate, A. J., Mino-Kenudson, M., and Engelman, J. A. Genotypic and histological evolution of lung cancers acquiring resistance to EGFR inhibitors. Sci Transl Med, *3:* 75ra26, 2011.
365. Courtney, K. D., Corcoran, R. B., and Engelman, J. A. The PI3K pathway as drug target in human cancer. J Clin Oncol, *28:* 1075-1083, 2010.
366. Wong, K. K., Engelman, J. A., and Cantley, L. C. Targeting the PI3K signaling pathway in cancer. Curr Opin Genet Dev, *20:* 87-90, 2010.
367. von Bueren, A. O., Shalaby, T., Rajtarova, J., Stearns, D., Eberhart, C. G., Helson, L., Arcaro, A., and Grotzer, M. A. Anti-proliferative activity of the quassinoid NBT-272 in childhood medulloblastoma cells. BMC Cancer, *7:* 19, 2007.
368. Loboda, A., Nebozhyn, M., Klinghoffer, R., Frazier, J., Chastain, M., Arthur, W., Roberts, B., Zhang, T., Chenard, M., Haines, B., Andersen, J., Nagashima, K., Paweletz, C., Lynch, B., Feldman, I., Dai, H., Huang, P., and Watters, J. A gene expression signature of RAS pathway dependence predicts response to PI3K and RAS pathway inhibitors and expands the population of RAS pathway activated tumors. BMC Med Genomics, *3:* 26.
369. Bender, A., Opel, D., Naumann, I., Kappler, R., Friedman, L., von Schweinitz, D., Debatin, K. M., and Fulda, S. PI3K inhibitors prime neuroblastoma cells for chemotherapy by shifting the balance towards pro-apoptotic Bcl-2 proteins and enhanced mitochondrial apoptosis. Oncogene, *30:* 494-503.
370. NCT00811993, C. g. I. A Study of R1507 in Combination With Multiple Standard Chemotherapy Treatments in Patients With Advanced Solid Tumors. First Received on December 18, 2008. Last Updated on March 15, 2011.
371. Yang, N., Huang, J., Greshock, J., Liang, S., Barchetti, A., Hasegawa, K., Kim, S., Giannakakis, A., Li, C., O'Brien-Jenkins, A., Katsaros, D., Butzow, R., Coukos, G., and Zhang, L. Transcriptional regulation of PIK3CA oncogene by NF-kappaB in ovarian cancer microenvironment. PLoS One, *3:* e1758, 2008.
372. Guerreiro, A. S., Boller, D., Doepfner, K. T., and Arcaro, A. IGF-IR: potential role in antitumor agents. Drug News Perspect, *19:* 261-272, 2006.

8 APPENDIX - REVIEW ARTICLE

TARGETING PHOSPHOINOSITIDE 3-KINASE SIGNALLING IN LUNG CANCER

Anna Wojtalla and Alexandre Arcaro

Crit Rev Oncol Hematol. 2011 Nov;80(2):278-90. doi: 10.1016/j.critrevonc.2011.01.007. Epub 2011 Feb 11. Review

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Critical Reviews in Oncology/Hematology 80 (2011) 278–290

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Targeting phosphoinositide 3-kinase signalling in lung cancer

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Accepted 18 January 2011

Contents

Abstract

Lung cancer is the leading cause of cancer-related mortality worldwide and more than 1 million people annually die in consequence of lung cancer. Although an improvement in lung cancer treatment could be achieved, especially in the last decade, the development of additional therapeutic strategies is urgently required in order to provide improved survival benefit for patients. Lung cancer formation is caused by genetic modifications commonly caused by tobacco smoking. Numerous studies have demonstrated the role of extracellular growth factors in lung cancer cell proliferation, metastasis, and chemoresistance. Mutations and amplifications in molecules related to receptor tyrosine signalling, such as EGFR, ErbB2, c-Met, c-Kit, VEGFR, PI3K, and PTEN are only some of the alterations known to contribute to the development of lung cancer. The phosphoinositide 3-kinase (PI3K) pathway, fundamental for cell development, growth, and survival, is known to be frequently altered in neoplasia, including carcinomas of the lung. Based on the high frequency of alterations, which include mutations and amplifications, leading to over-activation of certain upstream/downstream mediators, targeting components of the PI3K signalling pathway is considered to be a promising therapeutic approach in cancer treatment. In this article we will summarize the current knowledge about the involvement of PI3K signalling in lung cancer and discuss the development of targeted therapies involving PI3K pathway inhibitors.

Keywords: Non-small cell lung cancer; Small cell lung cancer; Phosphoinositide 3-kinase; Akt; Mammalian target of rapamycin

1. Introduction – lung cancer

Lung cancer is still the leading cause of cancer-related mortality in the Western world and every year approximately 1.4 million people are diagnosed with lung cancer [1]. Most commonly, lung cancer development is related to multiple genetic changes caused by exposure to carcinogens, for instance those found in tobacco smoke. Lung cancer can be divided into two main subtypes – non-small cell lung carcinoma (NSCLC) and small-cell lung carcinoma (SCLC). These subtypes differ not only in frequency of occurrence and prognosis, but also in biological behaviour, histological background, and characteristic genetic alterations.

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doi:10.1016/j.critrevonc.2011.01.007

Non-small cell lung cancer accounts for approximately 85% of all lung cancer cases, and is commonly related to tobacco smoking. In 9–15% of cases it is caused by exposure to other carcinogenic factors, such as polycyclic hydrocarbons, asbestos, or radon. Squamous cell carcinoma (SCC), adenocarcinoma (AC), and large cell lung carcinoma (LCLC) are the three most common types of NSCLC. The current treatment of NSCLC includes surgery, radiotherapy, and platinum-based chemotherapy. At the time of presentation at least 40% of patients are diagnosed with an advanced stage of disease [2], whereas less than 25% show early stage disease (stage I) [3]. Surgery, with lobectomy or pneumonectomy being the most common surgical resections, remains the standard of care for patients with early stage NSCLC able to safely undergo surgery [3]. The treatment of advanced NSCLC, which is mostly inoperable and therefore incurable, is aimed at controlling the disease to prolong life and sustain life quality, and commonly includes a combination of radio and chemotherapy. An increasing progress in the optimisation of chemotherapeutic regimens in combination with targeted therapies against individually activated oncogenes has led to an improvement of NSCLC outcome, but the median 5-year survival rate nowadays is still only 15% [4].

Small cell lung cancer accounts for approximately 15% of all lung cancer cases and is almost entirely related to tobacco smoking [5]. It is characterized by rapid growth and early metastasis. Thus, surgical resection is rarely possible. Chemotherapy with etoposide and platinum-based agents and in some cases also radiotherapy remain as the treatment options of choice [5–7]. Although SCLC initially responds to therapy most of the patients relapse. Due to metastasis and resistance to chemotherapy the clinical outcome is very poor and SCLC patients show an overall 5-year survival of less than 5% [6].

The molecular mechanisms responsible for lung cancer formation have been extensively studied. A striking event in the development of cancer is the escape of atypical cells from the normal growth control turning into a malignant and invasive phenotype. A growing body of evidence suggests that receptor tyrosine kinases (RTK) and their downstream effectors possess a high oncogenic capacity and that lung tumours selectively up-regulate different signalling pathways involving RTKs. The deregulation of survival pathways downstream of several RTK, such as EGFR, c-Met, c-Kit, VEGFR or IGF-1R is associated with lung cancer progression [8,9]. Deregulation of these pathways results in a lack of response to negative growth regulatory signals and the continuous presence of positive signals involving the PI3K/Akt pro-survival pathway that controls growth, motility and invasion.

2. Introduction – PI3K/Akt signalling

The phosphoinositide 3-kinase (PI3K) pathway, fundamental for cell development, growth, and survival, is known to play an important role in development of neoplasia. Its deregulation and contribution to carcinogenesis has been well documented and reviewed in the past, including in carcinomas of the lung. The PI3Ks are lipid kinases, which can be activated downstream of receptor tyrosine kinases (RTKs). The binding of specific growth factors to their corresponding receptors leads to activation of several downstream signalling cascades through important mediators, such as PI3K/Akt/mTOR or Ras/Raf/MEK/Erk, triggering signals to promote cell proliferation, prevent apoptosis, and increase cell migration. Targeting different molecules of the RTK/PI3K pathway with pharmacological inhibitors has already been shown to be a promising approach in cancer treatment [10–12]. Inhibitors of the RTK/PI3K pathway have reached the clinical stage in some cases and the development of new small molecule inhibitors is still an ongoing process.

Activation of PI3K takes place by binding of various growth factors to their specific receptors. Class I_A PI3K are recruited to RTKs at the plasma membrane as heterodimers, consisting of a regulatory (p85; Fig. 1) and a catalytic subunit (p110; Fig. 1). The p85 regulatory subunit binds to phosphotyrosine residues in the cytoplasmic domain of RTKs through its Src-homology 2 (SH2) domains, which leads to PI3K activation. Then the second messenger phosphatidylinositol-3,4,5-trisphosphate (PIP_3) is generated through phosphorylation of phosphatidylinositol-4,5-bisphosphate (PIP_2). A direct antagonist of PI3K is the phosphatase and tensin homologue deleted on chromosome 10 (PTEN). PTEN directly reverses the activity of PI3K by dephosphorylating PIP_3 into PIP_2 and therefore plays an important role as a negative controlling element of incoming signals. PIP_3 transduces activating signals by binding to pleckstrin homology (PH) domains of proteins, thereby recruiting them to the cell membrane. One centrally important downstream mediator of the PI3K signalling cascade is the serine threonine kinase Akt. Akt is recruited to the membrane via PIP_3 which is binding to its PH domain, followed by phosphorylation leading to its activation by PDK1 (3-phosphoinositide-dependent kinase 1) at threonine 308 and at serine 473 by mTORC2 (mammalian target of rapamycin complex 2). Activated Akt then mediates signals promoting cellular growth and survival and suppresses pro-apoptotic signals. Akt phosphorylates several intracellular proteins, including forkhead box O transcription factors (FoxO), the BCL2-associated agonist of cell death (BAD), and the glycogen synthase kinase 3 (GSK3), to promote cell cycle entry and cell survival. The proteins TSC1 (Hamartin) and TSC2 (Tuberin) form a complex that inhibits the activity of the small G-protein ras homologue enriched in brain (Rheb), which is necessary for mTORC1 activation. The Akt-mediated phosphorylation of TSC2 releases Rheb from its inhibited state. Rheb then accumulates in GTP-bound state and can directly activate mTORC1, which phosphorylates the p70S6 kinase (S6K1) and the eukaryotic translation initiation factor 4E binding protein 1 (4EBP1), leading to increased protein translation (Fig. 1).

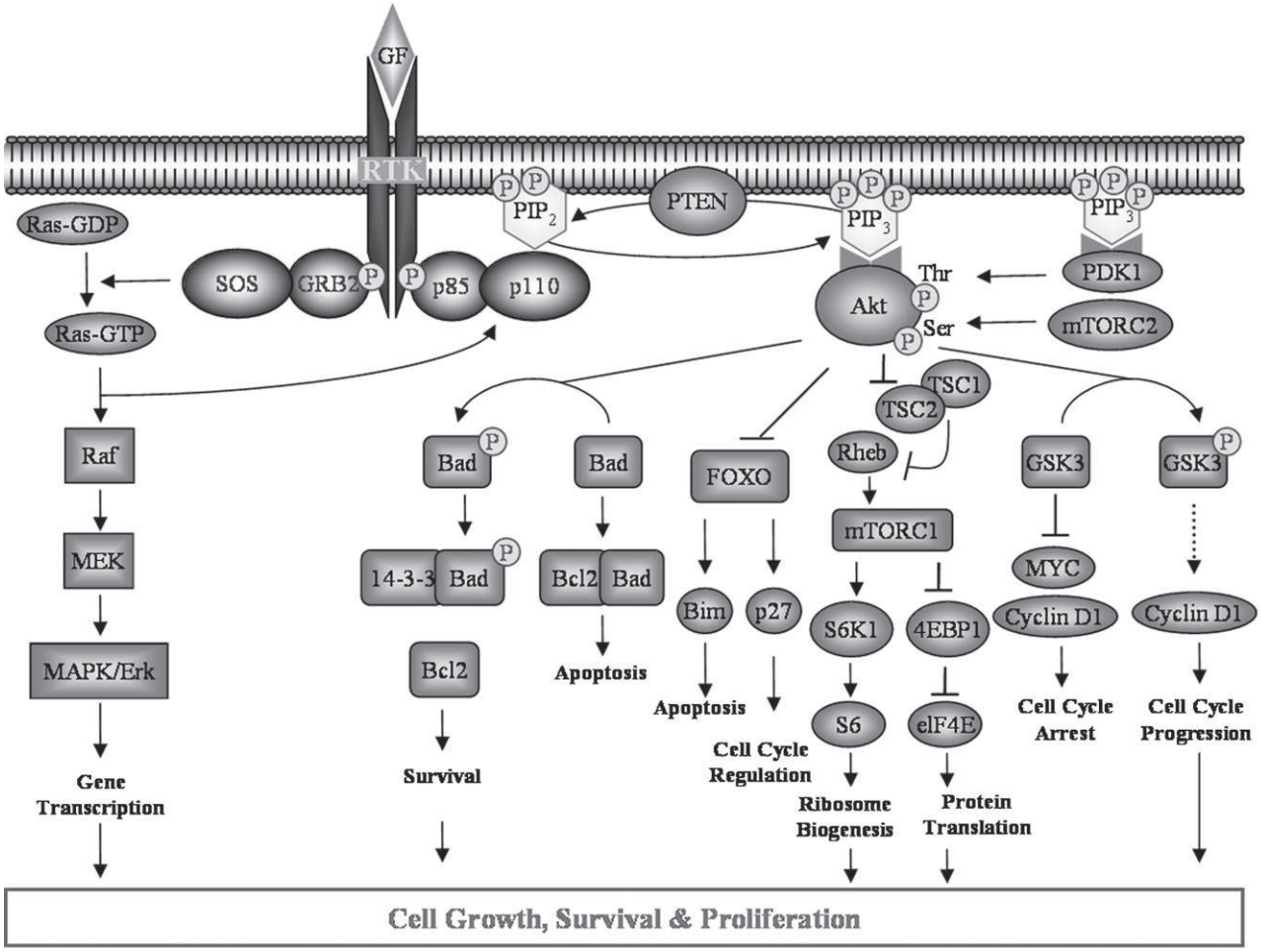

Fig. 1. PI3K Signalling.

3. Tobacco smoking and PI3K signalling activation

Lung cancer development is in most of the cases related to smoking, thus, raising the question whether components of tobacco smoke are involved and which pathways are activated during the carcinogenic processes. As a traditional model of tobacco-related tumourigenesis the theory has been established that tobacco components promote carcinogenesis through multistep processes which lead to the accumulation of mutations in key genes, such as *TP53* or *KRAS*. These genetic aberrations may cause tumour formation through circumvention of cell damage-induced cell death. Additionally, nicotine and the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NKK), both important components of tobacco smoke, were associated with early biochemical events occurring in cells exposed to tobacco smoke [13–15]. In an *in vitro* model of normal primary human airway epithelial cells (i) normal human bronchial epithelial cells (NHBEs), precursor of SCC; (ii) small airway epithelial cells (SAECs), precursor of AC activation of the PI3K/Akt pathway was demonstrated to be an early event promoting cellular survival due to exposure to nicotine and NKK, suggesting a molecular mechanism to overcome cell damage-induced apoptosis involved in lung cancer development [15]. Consistent with these results another study identified PI3K pathway activation in normal and premalignant bronchial airway epithelial cells of smokers with airway lesions, thus supporting the hypothesis that PI3K signalling activity is induced before the development of lung cancer [16]. Nicotine was also shown to increase cell proliferation and survival accompanied by activation of the PI3K/Akt/mTOR pathway in NSCLC and SCLC cells [13,14], and was associated with NFκB-dependent resistance to chemotherapeutic drugs [14]. Expression of nicotinic acetylcholine receptors (nAChRs), which respond to nicotine and NKK and activate Akt through PI3K, was found in normal and in lung cancer cells [13–15]. Another group reported that nicotine increased the expression of peroxisome proliferator-activated receptors (PPARβ/δ), which belong to the nuclear hormone receptor superfamily of ligand-dependent transcription factors, in a nAChR-mediated PI3K/Akt/mTOR-dependent manner, and thereby promoted NSCLC cell growth [17]. Furthermore, PI3K/Akt activation in response to nicotine exposure led to phosphorylation of

Bax through Akt, shortening its half-life and abrogating the pro-apoptotic activity of Bax. These events resulted in the promotion of cell survival [18]. Bad, another pro-apoptotic member of the Bcl-2 family, was shown to undergo multi-site phosphorylation involving nicotine-induced activation of the MAPKs Erk1/2, PI3K/Akt, and protein kinase A (PKA), leading to suppression of apoptosis [19]. Both studies could show that inhibition of the PI3K/Akt pathway abrogated nicotine-triggered anti-apoptotic signals and blocked lung cancer cell growth, suggesting that PI3K/Akt activation is an important step in nicotine-induced cancer cell survival [18,19].

4. The role of PI3K signalling in non-small cell lung cancer

Over-expression of both the regulatory subunit p85 and the catalytic p110α subunits of PI3K was demonstrated in primary lung carcinomas and their metastases, but not in normal lung tissue samples and was linked to lower grades of tumour differentiation [20]. Additionally, genomic amplification of *PIK3CA* was shown to occur in a substantial number of NSCLC tumours (70% squamous cell carcinoma, 38% large cell carcinoma, 19% adenocarcinoma) and pre-invasive lesions, suggesting the involvement of the PI3K/Akt pathway in early lung cancer progression [21]. However, mutations in the *PIK3CA* gene seem to be rare in NSCLC (∼2% primary tumours; ∼5% NSCLC cell lines) [22]. Downstream mediators of the PI3K/Akt pathway, like Akt, mTOR, or the tumour suppressor PTEN, are also genetically (and epigenetically) altered or deregulated in NSCLC, which supports the importance of the PI3K/Akt pathway for proliferative and anti-apoptotic signalling in NSCLC. Several groups have reported that Akt can be activated by either amplification of upstream molecules of the PI3K pathway, or directly through over-expression or over-activation of the Akt protein itself. Notably, *EGFR* mutations led to activation of anti-apoptotic pathways through Akt and Stat pro-survival signalling, but not Erk signalling [23,24]. Expression of all three isoforms of Akt (Akt1/2/3) was present in normal and tumour tissue of the lung [25]. Only 2.5% of NSCLC tumours harboured mutations in the *AKT2* gene, which could contribute to NSCLC tumourigenesis [26]. Therefore, deregulation of the pathway seems to occur more frequently at the post-transcriptional level. PI3K-dependent constitutively active Akt was reported in a panel of NSCLC cell lines and correlated with cellular survival and resistance to chemotherapy [27]. Increased activation (phosphorylation) of Akt was also reported in pre-malignant and malignant human bronchial epithelial (HBE) cells, but not in normal bronchial cells [28]. Additionally, high levels of phosphorylated Akt (SCC 68%; AC 62%) were detected in tumour tissue samples by immunohistochemistry [21,29–31], supporting PI3K pathway activation as a progressive event in lung tumourigenesis. Activation of Akt was also observed in primary NSCLC lung tumours, suggesting a role of activated Akt in the conversion of a pre-malignant lesion to lung cancer [29,30,32]. Moreover, over-expression or activation of Akt was reported to be a poor prognostic factor for NSCLC patients with primary tumours or stage I disease [33,34]. Several studies have shown that Akt over-activation is associated with low expression of the tumour-suppressor PTEN and that cells in which the *PTEN* gene is deleted or its expression is down-regulated display constitutively activated PI3K signalling [35–40]. PTEN plays an important role as negative regulator of the PI3K/Akt signalling acting as direct antagonist of PI3K. Mutations or homozygous deletion of the *PTEN* gene frequently occur in many different cancers but are rare in NSCLC [41–44]. Instead, reduced protein or complete loss of PTEN protein expression (70%) has been described in several studies [35,37]. The hypothesis of loss of heterozygosity and epigenetic alterations, such as promoter hypermethylation as regulators of PTEN expression in lung cancer was replaced by the idea of transcriptional or translational mechanisms, but the exact molecular mechanism still remains elusive [35]. Another downstream target of PI3K signalling, the mammalian target of rapamycin (mTOR), was shown to be activated in lung cancer cell lines [32,45] and interestingly, activation occurred more frequently in tumours with genetic alterations, such as *EGFR* mutations or PI3K/Akt over-expression [46]. Furthermore, mTOR phosphorylation was shown to increase with malignant progression and was demonstrated to be implicated in the metastatic development of NSCLC in *KRAS*-mutated models [47]. Additionally, it was shown that PI3K/Akt signalling contributes to chemo and radiotherapy resistance [27] and that, conversely, NSCLC cells with resistance to the tyrosine kinase inhibitor gefinitib show an increased PI3K/Akt activation [5,39]. Accordingly, targeting the PI3K/Akt pathway in NSCLC was suggested to be a promising approach to impair survival, chemoresistance, and metastasis. Manipulation of Akt activity by inhibition of PI3K with the small molecule inhibitors LY294002 or wortmannin, or transfection of kinase-dead Akt into cells with highly active Akt dramatically increased NSCLC cell sensitivity to chemotherapy- or radiation-induced apoptosis [24,27]. Plant flavonoids, such as deguelin were shown to selectively block Akt activity in a PI3K-dependent or -independent manner, thereby attenuating the activity of Bcl-2 by increasing the expression of proapoptotic Bax [28]. In genetic mouse models of oncogenic *KRAS*-induced lung cancer bronchioalveolar stem cells (BACS), expansion and malignant progression could be blocked by pharmacological inhibition of PI3K with PX866 and enhanced by genetic inactivation of *PTEN*, suggesting PI3K as a critical mediator of BACS expansion [48]. In NSCLC models carrying an oncogenic *KRAS* mutation, NVP-BEZ235, a dual pan-class I PI3K, mTORC1 and mTORC2 inhibitor, had anti-proliferative effects *in vitro* and *in vivo* and sensitized the tumour cells to the pro-apoptotic effects of radiotherapy [49]. Another group reported that murine lung cancers driven by mutant *KRAS* did not substantially respond to single-agent BEZ235 alone. No tumour shrinkage was observed,

even though decreased Akt phosphorylation was observed by Western blot and immunohistochemical analysis. Combined inhibition of PI3K (BEZ235) and MEK (ARRY-142886) however, resulted in marked synergistic tumour regression in this *KRAS*-mutant lung cancer [50]. In the same study, a mouse model of lung adenocarcinoma, initiated and maintained by expression of a somatic mutation in the p110α kinase domain (H1047R), showed marked tumour regression after treatment with the PI3K pathway inhibitor BEZ235 [50]. Therefore, PI3K inhibition could be a promising approach in lung cancer tumours driven by *PIK3CA* mutations, and, in combination with MEK inhibitors, it could also be useful in *KRAS*-mutated lung cancers, suggesting that *KRAS*-mutant lung cancers depend on both PI3K and MAPK signalling [50]. This hypothesis was confirmed by another group using a chemo-genomics approach in a panel of 84 NSCLC cell lines and associating specific genetic alterations with PI3K or MAPK pathway activation [51]. It was shown that tumours with genetically activated RTKs depend on PI3K signalling, whereas mutations in the *RAS/RAF* axis lead to a dependency on MAPK signalling [51]. A study in NSCLC models with activating mutations in the epidermal growth factor receptor (*EGFR*) could not detect induction of apoptosis when cells were treated with the dual PI3K/mTOR inhibitor BEZ235, in contrast to *HER2*-amplified breast cancers. However, apoptosis was induced in these *EGFR*-addicted cancers through simultaneous inhibition of PI3K/mTOR (BEZ235) and MEK (AZD6244) *in vitro* and tumour shrinkage could be observed *in vivo*, suggesting that simultaneous inhibition of PI3K/mTOR and MEK/ERK pathways could also be effective in *EGFR*-addicted lung cancers [52]. Another study showed that inhibition of mTOR signalling with rapamycin alone induced the pro-survival PI3K/Akt pathway in NSCLC cells, whereas LY294002 suppressed this effect, supporting the importance of PI3K in NSCLC [53]. It was also reported that the PI3K and MKK4/JNK (mitogen-activated protein kinase kinase-4/c-Jun NH_2-terminal kinase)-dependent pathways cooperate to maintain cell survival in wild type *PTEN* NSCLC cells and that simultaneous inhibition of both pathways increased apoptosis compared to targeting one pathway alone [54,55]. Accordingly, another group reported activator protein-1 (AP1) and PI3K/Akt-dependent growth in a panel of NSCLC cells. In this study simultaneous inhibition using a double-negative mutant of c-Jun (TAM67) and LY294002, did not result in the induction of apoptotic signals [56]. Taken together, these data suggest that up- and de-regulation of the PI3K/Akt pathway play a significant role in development, growth, and chemoresistance of NSCLC, but also indicate the importance of closely related pathways.

5. The role of PI3K signalling in small cell lung cancer

Small cell lung cancer (SCLC) accounts for approximately 15% of all lung cancers, with 90–95% of affected individuals dying of the disease within 5 years [5]. This type of lung cancer is initially a highly chemotherapy- and radiotherapy-responsive disease. However, the initial therapeutic response is followed by relapse and progressive development of chemotherapy resistance, and therefore the outcome is still very poor [57,58]. Numerous genetic and molecular alterations have been reported to be associated with the development of SCLC, including autocrine signalling loops, oncogene activation and loss of tumour-suppressor genes [5]. These genetic and molecular aberrations result in a lack of response to negative growth regulatory signals and the continuous presence of positive signals that regulate growth, motility, and invasion. Mutation in the *PIK3CA* gene, known as one of the most common genetic alterations present in human cancers, were found in primary SCLC (13%) and in SCLC cell lines (23%) [59]. Another study performed in 2008 failed to detect any mutations in the exons 9 and 20 of *PIK3CA*, but reported *PIK3CA* copy number gains (4.7%), which correlated with higher expression of activated Akt in SCLC cell lines [22]. Chromosome 3q26-ter amplification including the *PIK3CA* gene locus was also shown in 67% of SCLC tumour samples [21]. A recently published study showed that genes encoding components of the RTK/PI3K/mTOR axis and apoptosis-regulating proteins harbour high frequencies of copy number alterations in both SCLC tumours and SCLC cell lines [60]. Copy number gains could be identified in the *PIK3CA* gene in 76% of SCLC tumours and in 54% of the SCLC cell lines. The *AKT1* gene was amplified in 64% of tumours and 39% of cell lines. In addition, the gene *FRAP1* encoding the downstream target mTOR was shown to be amplified in more than 50% of SCLC tumours. In view of the loss of *PTEN* in 76% of SCLC tumours, the members of the PI3K/Akt pathway were suggested to be potential drug targets of SCLC in this study [60]. PTEN plays an important role as negative regulator of the PI3K/Akt signalling acting as direct antagonist of PI3K and is mutated (∼15%) or can be deleted in SCLC [42–44]. Constitutively activated PI3K was found in SCLC cell lines and shown to promote growth and anchorage-independence due to high levels of basal Akt and $p70^{s6k}$ activity in the late 1990s [61]. Differential over-expression of several PI3K isoforms and their contribution to Akt activation were demonstrated in different SCLC cell lines. In particular, cells over-expressing the PI3K subunit p110α showed enhanced Akt activation after growth factor stimulation. PTEN down-regulation could not be observed as a plausible reason for the higher Akt activity [62]. Additionally, in tumour tissue samples from SCLC patients high levels of phosphorylated Akt (∼50–70%) were detected by immunohistochemistry, supporting the involvement of the activated pathway in disease progression [21,63]. Other studies related integrin-induced activation of PI3K/Akt signalling and adhesion on extra cellular matrix (ECM) with resistance to various therapies and protection from apoptosis [15,64]. Adherent-growing SCLC cells, which are those thought to initially survive chemotherapy, were reported to activate Akt and thus to increase chemo and radiotherapy

resistance by mechanisms involving over-expression of anti-apoptotic proteins [65]. One possible mechanism by which SCLC cells can escape the effects of cytotoxic drugs was discovered in experiments elucidating SCLC responses to cisplatin, a DNA-damaging agent. Surprisingly, treatment with cisplatin up-regulated Akt activation and contributed to the expression of pro-survival proteins in SCLC cells [66].

Several autocrine loops have been described in SCLC cells, including stem cell factor (SCF)/c-Kit, insulin-like growth factor-I (IGF-I)/IGF-IR, and hepatocyte growth factor (HGF)/c-Met, which all lead to the activation of PI3K/Akt signalling and promote cell growth, survival, and chemotherapy resistance. Direct inhibition of the PI3K/Akt pathway with the PI3K-specific inhibitor LY294002 attenuated these effects on cell growth, led to apoptosis, and enhanced the apoptotic effects of chemotherapeutic agents, such as cisplatin or etoposide [64,67]. The same effect was observed after inhibition of Akt by expression of a dominant-negative mutant of this protein in SCLC cells [67]. Inhibition of Akt with the small-molecule inhibitor tricribine (known as AKT/protein kinase B signalling inhibitor 2) resulted in significantly affected growth and colony formation and pointed out a higher sensitivity of *PIK3CA*-mutated SCLC cells compared to *PIK3CA* wild-type cells [59]. Moreover, the use of specific RTK inhibitors for c-Met, c-Kit, IGF-IR [68–71], alkaloids (naltrindole; opioid receptor antagonist) [72] or antibodies [73] were reported to impair SCLC cell survival in a PI3K/Akt-dependent manner.

6. Targeting the PI3K signalling in lung cancer – PI3K pathway inhibitors in clinical trials

Targeting PI3K signalling with pharmacological inhibitors has become an important experimental therapeutic approach. Targeting the RTK/PI3K/Akt cell survival axis and its downstream mediators with small molecule inhibitors (tyrosine kinase inhibitors, TKIs) is, beside treating cancer with classical chemotherapeutical agents, one of the most prominent approaches used in cancer therapy and has been reviewed in the past for various human cancers. The use of specific TKIs has led to a progress in cancer treatment options, especially in cancer types carrying a particular oncogene addiction, which leads to dependency on the activity of one particular tyrosine kinase. In lung cancer the knowledge about molecular alterations has predicted the use of TKIs, which are nowadays used in the clinics. However, the recurrence and metastasis of tumours, which are associated with a switch in the oncogene addiction, changing in signalling pathway activation, followed by the development of chemoresistance have raised the awareness about other pathways and/or multi-point intervention to target different signalling nodes in parallel. Compared to the development and the use of TKIs in the clinics, the development of PI3K inhibitors appears to be still in an early phase, but is rapidly processing. Looking at current or soon initiating clinical trials in various cancer types, it is clear that PI3K inhibitors have arrived to the clinical stage. Below, we will describe various PI3K pathway inhibitors, which have reached clinical trials for the potential treatment of NSCLC and SCLC.

The orally available selective class I PI3K inhibitor GDC-0941 was identified to be a potent agent for the treatment of cancer [74] and will soon enter phase I, open-label, dose-escalation studies for the treatment of patients with advanced NSCLC, in combination with paclitaxel and carboplatin, with or without the monoclonal VEGF-antibody bevacizumab (Table 1) [75], and in combination with the EGFR inhibitor erlotinib (Table 1) [76]. GDC-0941 was already tested in phase I clinical trials in patients with advanced solid tumours

Table 1
Selection of clinical trials targeting PI3K signalling with emphasis on lung cancer.

Compound	Trial	Cancer	Mechanism	Reference
GDC-0941 + paclitaxel + carboplatin with/without bevacizumab	Phase I; recruiting	NSCLC	Class I PI3K inhibitor	Study NCT00974584 [75]
GDC-0941 + erlotinib	Phase I; recruiting	NSCLC + others	Class I PI3K inhibitor	Study NCT00975182 [76]
GDC-0941	Phase I	Solid tumours	Class I PI3K inhibitor	Von Hoff et al. [78]
GDC-0941	Phase I	Solid tumours	Class I PI3K inhibitor	Baird et al. [77]
XL147	Phase I	NSCLC + others	Class I PI3K inhibitor	Edelmann et al. [87]
XL147 + paclitaxel and carboplatin	Phase I; recruiting	NSCLC	Class I PI3K inhibitor	Study NCT00756847 [85]
XL147 + erlotinib	Phase I; recruiting	NSCLC	Class I PI3K inhibitor	Study NCT00692640 [86]
BEZ235	Phase I	Solid tumours	PI3K + mTOR inhibitor	Burris et al. [111]
rad001 (Everolimus) + docetaxel	Phase I	NSCLC	mTOR inhibitor	Ramalingam et al. [93]
RAD001 (Everolimus)	Phase II	NSCLC	mTOR inhibitor	Soria et al. [95]
RAD001 (Everolimus), carboplatin, etoposide	Phase I; recruiting	SCLC + others	mTOR inhibitor	Study NCT00807755 [107]
RAD001 (Everolimus) + paclitaxel	Phase I; recruiting	SCLC	mTOR inhibitor	Study NCT01079481 [108]
RAD001 (Everolimus)	Phase II; ongoing	SCLC	mTOR inhibitor	Study NCT00374140 [109]
TS-1, cisplatin (CDDP) and RAD001 (Everolimus)	Phase I; not yet recruiting	Solid tumours	mTOR inhibitor	Study NCT01096199 [98]
Temsirolimus + vinorelbine ditartrate	Phase I; recruiting	SCLC + others	mTOR inhibitor	Study NCT01155258 [105]
Metformin and temsirolimus	Phase I; recruiting	SCLC + others	mTOR inhibitor	Study NCT00659568 [106]
MK-2206 + gefinitib	Phase I; not yet recruiting	NSCLC	Akt inhibitor	Study NCT01147211 [117]

(breast, ovarian, melanoma) and was generally well tolerated. GDC-0941 treatment resulted in effects on PI3K pathway activation shown by decreased levels of pAkt and pS6 in tumour biopsies, and signs of anti-tumour activity could be observed (Table 1) [77,78]. In a preclinical model of *HER2*-amplified breast cancer cells, which often show resistance to HER2-targeted therapy due to PI3K pathway activation, GDC-0941 showed significant activity in more than 70% of breast cancer cell lines. In combination with the HER2-specific antibodies trastuzumab and pertuzumab treatment with GDC-0941 resulted in enhanced growth inhibition and suppression of the Akt and MAPK pro-survival pathways [79]. In another study GDC-0941 inhibited the cell proliferation of both trastuzumab-sensitive and -insensitive cells and induced apoptosis in combination with trastuzumab [80]. Additionally, in a trastuzumab-insensitive breast cancer xenograft model single-agent GDC-0941 could inhibit the growth of a *HER2*-amplified tumour harbouring *PIK3CA* mutation, whereas combined treatment with GDC-0941 and trastuzumab did not result in significant benefit [80]. Inhibition of the PI3K pathway and tumour growth could be observed also in various xenograft models of breast cancer [81,82], lung cancer, glioblastoma [74,83], ovarian cancer [83], and prostate cancer [82]. Furthermore, oncogenic alterations, such as activating mutations in the *PIK3CA* gene and amplification of the *HER2* gene, could be identified as molecular predictors for the sensitivity of GDC-0941-response *in vitro* and *in vivo*, suggesting PI3K signalling addiction in tumours harbouring these alterations and therefore being promising candidates for PI3K-targeted therapies [84].

XL147 (SAR245408) is a selective inhibitor of class I PI3K and will enter a phase I, dose-escalation study in solid tumours, such as NSCLC, ovarian cancer, and endometrial cancer in combinatorial treatment with the accepted treatment regimen of paclitaxel and carboplatin (Table 1) [85] Another phase I study currently recruiting participants will evaluate the efficacy of XL147 in combination with the EGFR inhibitor erlotinib in patients with NSCLC or other solid tumours (Table 1) [86]. In a phase I dose-escalation study in patients with NSCLC, lymphoma and other solid tumours, daily treatment with XL147 resulted in a reduction in PI3K signalling and phosphorylation of MEK and ERK in tumour tissue samples, reduction in the target lesion, and prolonged stable disease (Table 1) [87].

RAD001 (Everolimus), a rapamycin derivative, is an orally available mTOR inhibitor. Various studies tested RAD001 as a monotherapy, or in combinatorial treatments in diverse tumours. Here we will focus on RAD001 in the treatment of lung cancer (Table 1). In preclinical studies RAD001 has shown anti-tumour activity in *in vitro* and *in vivo* models, including NSCLC [88–92]. In a phase I clinical trial, a combinatorial treatment of docetaxel and RAD001 was administered to patients with advanced NSCLC [93]. Docetaxel is a semisynthetic taxane and an approved treatment for advanced NSCLC, but was shown to cause activation of the PI3K/Akt/mTOR axis as a resistance mechanism to taxane therapy [94]. Following these observations, the hypothesis is to combine the treatment of docetaxel with RAD001 to overcome this resistance mechanism [93]. Twent-four patients with advanced stage NSCLC and progression after platinum-based chemotherapy were treated with escalating doses of docetaxel (intravenous, day 1) and everolimus (orally daily, days 1–19) in a 3-weeks treatment cycle until progression of disease. Sixteen patients received >1 cycle of therapy. One patient (adenocarcinoma) experienced a partial response with regression in the primary tumour mass. Ten patients experienced disease stabilisation. Overall, the combination of docetaxel and everolimus was feasible and demonstrated promising results in NSCLC patients [93]. RAD001 as single-agent treatment was investigated in a phase II clinical study in patients with advanced NSCLC previously treated with chemotherapy alone (stratum 1), or with chemotherapy and EGFR inhibitors (stratum 2). Patients received 10 mg everolimus daily. The PFS was 79 days (stratum 1) and 81 days (stratum 2) [95]. Patients showed partial response or disease stabilisation, demonstrating a modest efficacy for the treatment in patients in whom multiple lines of systemic therapy had failed [95]. Another phase I clinical trial employed the combinatorial treatment of gefinitib and everolimus in patients with advanced NSCLC to restore gefinitib sensitivity. Five or ten milligrams of everolimus and 250 mg gefinitib were given to 10 patients orally daily. The maximum tolerated dose in combination with 250 mg gefinitib was 5 mg everolimus and 2 patients experienced a partial response under these treatment conditions [96]. Based on these results, a following phase II clinical study assessed the efficacy of the combination of gefinitib and everolimus in patients with advanced NSCLC, either without prior chemotherapeutic treatment, or with platinum-based chemotherapy. Patients received 5 mg everolimus and 250 mg gefinitib daily. Partial responses were seen in 8 of 62 patients (response rate 13%) and the median time to progression was 4 months. The median overall survival was 12 months, 27 months for patients with no prior chemotherapy, and 11 month for patients previously treated with chemotherapy [97]. In another phase I clinical study RAD001 will be tested in patients with advanced, metastatic or recurrent solid malignancies in combination with TS-1/cisplatin therapy (Table 1) [98]. Up-regulation of the PI3K/Akt/mTOR pathway was found to be an important mechanism for resistance to cisplatin [66,99,100] and could be reversed by mTOR inhibition in lung cancer cells [101], suggesting a therapeutic approach consisting of platinum-based chemotherapy in parallel with inhibition of the PI3K/Akt/mTOR axis to overcome (acquired) cisplatin-resistance in human cancers.

In SCLC cell lines and tumour tissue samples of SCLC patients, mTOR expression was found to be over-expressed, compared to normal lung epithelial cells and normal lung tissue [102]. Additionally, RAD001 was able to inhibit cell growth in a panel of SCLC cell lines *in vitro* and also in a xenograft model of SCLC [102,103]. RAD001 down-regulated the basal and growth factor-stimulated activation

of downstream targets of mTOR, and sensitized SCLC cells to chemotherapy (etoposide) [102]. Furthermore, SCLC cells which displayed an enhanced or constitutive activation of the Akt/mTOR signalling were shown to be more sensitive to RAD001 treatment, suggesting mTOR inhibition as potential therapeutic target in patients with SCLC [102]. In a phase II clinical study, temsirolimus, another rapamycin derivative and mTOR inhibitor, was evaluated as a single-agent in SCLC patients after chemotherapy to study the progression free survival (PFS) and toxicities. Forty-four patients received 25 mg and 41 patients received 250 mg temsirolimus (CCI-779) intravenously every week until disease progression. The overall median PFS was 2.2 months and a difference between low dose and high dose treated patients could be observed only for males (median PFS for low dose: 1.7 months; median PFS for high dose arm: 3 months). One patient (1.2%) experienced a partial response and 6 patients (7.2%) stable disease, whereas 74 patients (89.2%) experienced progressive disease. The median overall survival was 8 months, with a significant difference between low dose (6.6 months) and high dose (9.5 months) treatment. In conclusion, temsirolimus given to responding or stable patients with extensive-stage SCLC after chemotherapy did not seem to result in a prolongation of PFS in these patients [104]. Currently, RAD001 is, beside a few clinical studies involving temsirolimus (Table 1) [105,106], the only PI3K pathway inhibitor which has entered clinical trials for patients with SCLC (Table 1) [107–109]. Two phase I dose-escalation studies are recruiting participants to test RAD001 in combinatorial treatment. A first trial will combine RAD001 with paclitaxel, and another one with etoposide or carboplatin, the chemotherapeutic drugs commonly used in SCLC patients (Table 1) [107,108]. In an ongoing phase II clinical study RAD001 is administered as a monotherapy to patients with previously treated SCLC to determine overall and progression-free survival, objective response, and toxicities (Table 1) [109]. This study was the first phase II trial evaluating single-agent everolimus in previously treated, relapsed SCLC. Everolimus (10 mg) was given orally daily to 40 patients until disease progression. Among 35 evaluable patients, all progressed. One patient showed partial response (3%), 8 (23%) stable disease. The median survival was 6.7 month and the median time to progression 1.3 month. Overall, in this study the duration until disease progression was relatively short and everolimus showed limited activity as a monotherapy [110].

BEZ235, an imidazo-quinoline derivative, is an orally available dual PI3K/mTOR inhibitor, which inhibits the kinase activity by binding to the ATP-binding site of both of the enzymes. In a first-in-human phase I study the treatment of patients with advanced, unresectable solid tumours (e.g., breast cancer and colorectal cancer) with single-agent BEZ235 resulted in anti-cancer activity, especially in patients with PI3K dysregulated tumours (Table 1) [111]. In preclinical studies its anti-tumour activity was shown already *in vitro* and *in vivo* [49,50,112–114]. In various cancer cell lines BEZ235 treatment resulted in a decrease in proliferation [49,50,113,114], in enhanced block of Akt activity, compared to the PI3K inhibitor LY294002, and in a G1 cell cycle arrest [113]. In xenograft models of these cancer types BEZ235 inhibited tumour growth in a dose-dependent manner [113]. Inhibition of PI3K and mTOR with BEZ235 also reversed hyperactivation of the PI3K/mTOR pathway in trastuzumab-resistant p110α-mutated (H1047R) breast cancer cells and reduced tumour growth in a H1047R xenograft [114]. Additionally, BEZ235 was shown to block growth of tumour cells more effectively *in vitro* and *in vivo* than single-inhibition of mTORC1 with rapamycin [112]. BEZ235 could also inhibit proliferative signalling in PI3K/Akt/mTOR-addicted lymphomas by suppression of autocrine and paracrine growth factors [115] and overcome the RAD001-induced negative feedback activation of Akt in neuroendocrine tumours [116].

Another phase I clinical trial will test MK-2206, an orally active inhibitor of human AKT1, AKT2, and AKT3, in combination with the EGFR inhibitor gefitinib in patients with advanced NSCLC tumours harbouring *EGFR* mutation (Table 1) [117]. In preclinical studies treatment with MK-2206 inhibited tumour cell proliferation synergistically with molecular targeted therapies, such as erlotinib or lapatinib, or cytotoxic agents, such as doxorubicin, docetaxel, and carboplatin *in vitro* and *in vivo* [118].

Taken together, targeting mTOR as a novel treatment strategy seems to show promising effects in NSCLC. In SCLC, comparable effects are still missing, but this might be because of fewer results of completed clinical trials. Generally, and due to the little amount of completed clinical studies, compared to the use of other targeted therapies in lung cancer, further clinical studies using targeted therapy of the PI3K signalling pathway are required to evaluate the pharmacokinetics and the anti-cancer activity of these agents.

Even though the potential advantages of targeting PI3K signalling seem to be obvious, judging from preclinical and clinical studies, it is also important to evaluate the potential critical toxicities and side effects for patients. By targeting components of a pathway controlling crucial signalling events of cell metabolism various adverse events are likely to happen. From different clinical studies in lung cancer patients treated with the rapamycin derivates everolimus or temsirolimus, it is known that patients experience randomly hematologic side effects, such as neutropenia and thrombocytopenia, and non-hematologic adverse events, such as fatigue, stomatitis, mucositis, nausea, infection, hyperglycemia, and pneumonitis [93,95,96,104,110]. The severity of the adverse events was also depending on whether everolimus was administered as a monotherapy or as part of a combination therapy, where toxicities were overlapping [96]. Two studies characterising the effects of everolimus or temsirolimus on lung toxicities in NSCLC patients reported drug-related pneumonitis in 25% and 36% in evaluated patients, respectively [119,120]. In a preclinical study, the inhibition of Akt1 and Akt2 with a specific small molecule resulted in transient insulin resistance and reversible, dose-dependent hyperglycemia and hyperinsulinemia [121]. Akt1 and Akt2

are known to play essential roles in insulin signalling and glucose homeostasis. Akt inhibitors have been shown to cause hyperglycemia in mice [122–124]. In addition, *AKT2* knock-out mice are glucose intolerant and show an insulin-resistant phenotype [125]. PI3K isoforms also play important roles in insulin signalling and glucose uptake. Double heterozygous knock-out of the PI3K catalytic subunits p110α and p110β led to impaired insulin responses in mice, resulting in slight glucose intolerance and hyperinsulinemia [126]. Mice carrying a heterozygous knock-in mutation (D933A) that abrogates p110α kinase activity, showed hyperinsulinemia, glucose intolerance, and increased adiposity [127]. Additionally, the PI3K subunit p110δ plays an important role for the proper regulation of immune responses. Mice deficient in p110δ had severely impaired B and T cell function [128,129]. Mice expressing a catalytically inactive form of p110δ (p110δ^{D910A}) showed attenuated immune responses *in vivo* and the antigen receptor signalling in B and T cells was impaired [129]. The phenotypes described in gene-targeted mice may be used to extrapolate toxicities which are likely to occur upon inhibition of PI3K isoforms as a therapeutic strategy in human patients. However and surprisingly, in a first phase I study in cancer patients, BEZ235, a dual PI3K/mTOR inhibitor, was well tolerated and showed only mild or moderate adverse effects, such as nausea, vomiting, fatigue, and anorexia and no dose-limiting toxicities were observed [111]. In addition, other PI3K inhibitors, such as GDC-0941 or XL147, were reported to be well tolerated without or only a few DLTs [77,78,87].

7. Conclusion and perspective

The importance of the PI3K signalling pathway, which controls cell survival and proliferation processes, and its impact on cancer development has been demonstrated in the last two decades in various human neoplasms, including lung cancer. The escape of atypical cells from the normal growth control, in addition to deregulation of PI3K signalling and closely related pathways drives cells into a malignant and invasive phenotype. Deregulation of these pathways results in a lack of response to negative growth regulatory signals and the continuous presence of positive signals involving the RTK/PI3K/Akt pro-survival axis. Therefore, targeting RTK/PI3K/Akt signalling with small molecule inhibitors has become an interesting and promising approach in lung cancer therapy development. Indeed, there are now several targeted agents directed against the PI3K/Akt/mTOR pathway which have entered clinical trials in NSCLC and SCLC. The use of targeted therapies in addition to commonly administered chemotherapy could improve clinical benefit for patients, especially in patients with cancer types depending on the presence of one particular oncogene, such as EGFR in NSCLC. Additionally, even though the whole "cancer cell machinery" is not yet adequately understood and there exist many "missing links", the growing knowledge about genetic alterations driving cancer development and maintenance provides the possibility to identify predictive molecular markers, which are of importance for further therapy development and clinical benefit.

Conflict of interest statement

The authors declare no conflict of interest.

Reviewer

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Acknowledgement

This work was supported by a grant from the Wilhelm Sander-Stiftung.

References

[1] Jemal A, Siegel R, Xu J, et al. Cancer statistics, 2010. CA Cancer J Clin 2010.
[2] Vandenbroucke E, De Ryck F, Surmont V, et al. What is the role for surgery in patients with stage III non-small cell lung cancer? Curr Opin Pulm Med 2009;15(4):295–302.
[3] Robinson CG, Bradley JD. The treatment of early-stage disease. Semin Radiat Oncol 2010;20(3):178–85.
[4] Molina JR, Adjei AA, Jett JR. Advances in chemotherapy of non-small cell lung cancer. Chest 2006;130(4):1211–9.
[5] Jackman DM, Johnson BE. Small-cell lung cancer. Lancet 2005;366(9494):1385–96.
[6] Demedts IK, Vermaelen KY, van Meerbeeck JP. Treatment of extensive-stage small cell lung carcinoma: current status and future prospects. Eur Respir J 2010;35(1):202–15.
[7] Sculier JP, Berghmans T, Meert AP. Update in lung cancer and mesothelioma 2009. Am J Respir Crit Care Med 2010;181(8):773–81.
[8] Hodkinson PS, Mackinnon A, Sethi T. Targeting growth factors in lung cancer. Chest 2008;133(5):1209–16.
[9] Pisick E, Jagadeesh S, Salgia R. Receptor tyrosine kinases and inhibitors in lung cancer. ScientificWorldJournal 2004;4:589–604.
[10] Engelman JA. Targeting PI3K signalling in cancer: opportunities, challenges and limitations. Nat Rev Cancer 2009;9(8):550–62.
[11] Garcia-Echeverria C, Sellers WR. Drug discovery approaches targeting the PI3K/Akt pathway in cancer. Oncogene 2008;27(41):5511–26.
[12] Liu P, Cheng H, Roberts TM, et al. Targeting the phosphoinositide 3-kinase pathway in cancer. Nat Rev Drug Discov 2009;8(8):627–44.
[13] Carlisle DL, Liu X, Hopkins TM, et al. Nicotine activates cell-signaling pathways through muscle-type and neuronal nicotinic acetylcholine receptors in non-small cell lung cancer cells. Pulm Pharmacol Ther 2006.
[14] Tsurutani J, Castillo SS, Brognard J, et al. Tobacco components stimulate Akt-dependent proliferation and NFkappaB-dependent survival in lung cancer cells. Carcinogenesis 2005;26(7):1182–95.
[15] West KA, Brognard J, Clark AS, et al. Rapid Akt activation by nicotine and a tobacco carcinogen modulates the phenotype of normal human airway epithelial cells. J Clin Invest 2003;111(1):81–90.

[16] Gustafson AM, Soldi R, Anderlind C, et al. Airway PI3K pathway activation is an early and reversible event in lung cancer development. Sci Transl Med 2010;2(26):26ra25.
[17] Sun X, Ritzenthaler JD, Zhong X, et al. Nicotine stimulates PPARbeta/delta expression in human lung carcinoma cells through activation of PI3K/mTOR and suppression of AP-2alpha. Cancer Res 2009;69(16):6445–53.
[18] Xin M, Deng X. Nicotine inactivation of the proapoptotic function of Bax through phosphorylation. J Biol Chem 2005;280(11):10781–9.
[19] Jin Z, Gao F, Flagg T, et al. Nicotine induces multi-site phosphorylation of Bad in association with suppression of apoptosis. J Biol Chem 2004;279(22):23837–44.
[20] Lin X, Böhle AS, Dohrmann P, et al. Overexpression of phosphatidylinositol 3-kinase in human lung cancer. Langenbecks Arch Surg 2001;386(4):293–301.
[21] Massion PP, Taflan PM, Shyr Y, et al. Early involvement of the phosphatidylinositol 3-kinase/Akt pathway in lung cancer progression. Am J Respir Crit Care Med 2004;170(10):1088–94.
[22] Yamamoto H, Shigematsu H, Nomura M, et al. PIK3CA mutations and copy number gains in human lung cancers. Cancer Res 2008;68(17):6913–21.
[23] Akca H, Tani M, Hishida T, et al. Activation of the AKT and STAT3 pathways and prolonged survival by a mutant EGFR in human lung cancer cells. Lung Cancer 2006;54(1):25–33.
[24] Sordella R, Bell DW, Haber DA, et al. Gefitinib-sensitizing EGFR mutations in lung cancer activate anti-apoptotic pathways. Science 2004;305(5687):1163–7.
[25] Zinda MJ, Johnson MA, Paul JD, et al. AKT-1, -2, and -3 are expressed in both normal and tumor tissues of the lung, breast, prostate, and colon. Clin Cancer Res 2001;7(8):2475–9.
[26] Soung YH, Lee JW, Nam SW, et al. Mutational analysis of AKT1, AKT2 and AKT3 genes in common human carcinomas. Oncology 2006;70(4):285–9.
[27] Brognard J, Clark AS, Ni Y, et al. Akt/protein kinase B is constitutively active in non-small cell lung cancer cells and promotes cellular survival and resistance to chemotherapy and radiation. Cancer Res 2001;61(10):3986–97.
[28] Chun KH, Kosmeder II JW, Sun S, et al. Effects of deguelin on the phosphatidylinositol 3-kinase/Akt pathway and apoptosis in premalignant human bronchial epithelial cells. J Natl Cancer Inst 2003;95(4):291–302.
[29] Lee SH, Kim HS, Park WS, et al. Non-small cell lung cancers frequently express phosphorylated Akt; an immunohistochemical study. APMIS 2002;110(7–8):587–92.
[30] Tsao AS, McDonnell T, Lam S, et al. Increased phospho-AKT (Ser(473)) expression in bronchial dysplasia: implications for lung cancer prevention studies. Cancer Epidemiol Biomarkers Prev 2003;12(7):660–4.
[31] Tsurutani J, Steinberg SM, Ballas M, et al. Prognostic significance of clinical factors and Akt activation in patients with bronchioloalveolar carcinoma. Lung Cancer 2007;55(1):115–21.
[32] Balsara BR, Pei J, Mitsuuchi Y, et al. Frequent activation of AKT in non-small cell lung carcinomas and preneoplastic bronchial lesions. Carcinogenesis 2004;25(11):2053–9.
[33] David O, Jett J, LeBeau H, et al. Phospho-Akt overexpression in non-small cell lung cancer confers significant stage-independent survival disadvantage. Clin Cancer Res 2004;10(20):6865–71.
[34] Tsurutani J, Fukuoka J, Tsurutani H, et al. Evaluation of two phosphorylation sites improves the prognostic significance of Akt activation in non-small-cell lung cancer tumors. J Clin Oncol 2006;24(2): 306–14.
[35] Marsit CJ, Zheng S, Aldape K, et al. PTEN expression in non-small-cell lung cancer: evaluating its relation to tumor characteristics, allelic loss, and epigenetic alteration. Hum Pathol 2005;36(7):768–76.
[36] Singhal S, Amin KM, Kruklitis R, et al. Differentially expressed apoptotic genes in early stage lung adenocarcinoma predicted by expression profiling. Cancer Biol Ther 2003;2(5):566–71.
[37] Soria JC, Lee HY, Lee JI, et al. Lack of PTEN expression in non-small cell lung cancer could be related to promoter methylation. Clin Cancer Res 2002;8(5):1178–84.
[38] David O. Akt and PTEN: new diagnostic markers of non-small cell lung cancer? J Cell Mol Med 2001;5(4):430–3.
[39] Kokubo Y, Gemma A, Noro R, et al. Reduction of PTEN protein and loss of epidermal growth factor receptor gene mutation in lung cancer with natural resistance to gefitinib (IRESSA). Br J Cancer 2005;92(9):1711–9.
[40] Tang JM, He QY, Guo RX, et al. Phosphorylated Akt overexpression and loss of PTEN expression in non-small cell lung cancer confers poor prognosis. Lung Cancer 2006;51(2):181–91.
[41] Teng DH, Hu R, Lin H, et al. MMAC1/PTEN mutations in primary tumor specimens and tumor cell lines. Cancer Res 1997;57(23):5221–5.
[42] Forgacs E, Biesterveld EJ, Sekido Y, et al. Mutation analysis of the PTEN/MMAC1 gene in lung cancer. Oncogene 1998;17(12):1557–65.
[43] Forgacs E, Zöchbauer-Müller S, Oláh E, et al. Molecular genetic abnormalities in the pathogenesis of human lung cancer. Pathol Oncol Res 2001;7(1):6–13.
[44] Yokomizo A, Tindall DJ, Drabkin H, et al. PTEN/MMAC1 mutations identified in small cell, but not in non-small cell lung cancers. Oncogene 1998;17(4):475–9.
[45] Han S, Khuri FR, Roman J. Fibronectin stimulates non-small cell lung carcinoma cell growth through activation of Akt/mammalian target of rapamycin/S6 kinase and inactivation of LKB1/AMP-activated protein kinase signal pathways. Cancer Res 2006;66(1):315–23.
[46] Conde E, Angulo B, Tang M, et al. Molecular context of the EGFR mutations: evidence for the activation of mTOR/S6K signaling. Clin Cancer Res 2006;12(3 Pt. 1):710–7.
[47] Wislez M, Spencer ML, Izzo JG, et al. Inhibition of mammalian target of rapamycin reverses alveolar epithelial neoplasia induced by oncogenic K-ras. Cancer Res 2005;65(8):3226–35.
[48] Yang Y, Iwanaga K, Raso MG, et al. Phosphatidylinositol 3-kinase mediates bronchioalveolar stem cell expansion in mouse models of oncogenic K-ras-induced lung cancer. PLoS One 2008;3(5):e2220.
[49] Konstantinidou G, Bey EA, Rabellino A, et al. Dual phosphoinositide 3-kinase/mammalian target of rapamycin blockade is an effective radiosensitizing strategy for the treatment of non-small cell lung cancer harboring K-RAS mutations. Cancer Res 2009;69(19):7644–52.
[50] Engelman JA, Chen L, Tan X, et al. Effective use of PI3K and MEK inhibitors to treat mutant Kras G12D and PIK3CA H1047R murine lung cancers. Nat Med 2008;14(12):1351–6.
[51] Sos ML, Fischer S, Ullrich R, et al. Identifying genotype-dependent efficacy of single and combined PI3K- and MAPK-pathway inhibition in cancer. Proc Natl Acad Sci U S A 2009;106(43):18351–6.
[52] Faber AC, Li D, Song Y, et al. Differential induction of apoptosis in HER2 and EGFR addicted cancers following PI3K inhibition. Proc Natl Acad Sci U S A 2009;106(46):19503–8.
[53] Sun SY, Rosenberg LM, Wang X, et al. Activation of Akt and eIF4E survival pathways by rapamycin-mediated mammalian target of rapamycin inhibition. Cancer Res 2005;65(16):7052–8.
[54] Lee HY, Oh SH, Suh YA, et al. Response of non-small cell lung cancer cells to the inhibitors of phosphatidylinositol 3-kinase/Akt- and MAPK kinase 4/c-Jun NH2-terminal kinase pathways: an effective therapeutic strategy for lung cancer. Clin Cancer Res 2005;11(16):6065–74.
[55] Lee HY, Srinivas H, Xia D, et al. Evidence that phosphatidylinositol 3-kinase- and mitogen-activated protein kinase kinase-4/c-Jun NH2-terminal kinase-dependent pathways cooperate to maintain lung cancer cell survival. J Biol Chem 2003;278(26):23630–8.
[56] Kikuchi J, Kinoshita I, Shimizu Y, et al. Simultaneous blockade of AP-1 and phosphatidylinositol 3-kinase pathway in non-small cell lung cancer cells. Br J Cancer 2008;99(12):2013–9.
[57] Govindan R, Page N, Morgensztern D, et al. Changing epidemiology of small-cell lung cancer in the United States over the last 30 years:

analysis of the surveillance, epidemiologic, and end results database. J Clin Oncol 2006;24(28):4539–44.
[58] Cooper S, Spiro SG. Small cell lung cancer: treatment review. Respirology 2006;11(3):241–8.
[59] Shibata T, Kokubu A, Tsuta K, et al. Oncogenic mutation of PIK3CA in small cell lung carcinoma: a potential therapeutic target pathway for chemotherapy-resistant lung cancer. Cancer Lett 2009;283(2):203–11.
[60] Voortman J, Lee JH, Killian JK, et al. Array comparative genomic hybridization-based characterization of genetic alterations in pulmonary neuroendocrine tumors. Proc Natl Acad Sci U S A 2010.
[61] Moore SM, Rintoul RC, Walker TR, et al. The presence of a constitutively active phosphoinositide 3-kinase in small cell lung cancer cells mediates anchorage-independent proliferation via a protein kinase B and p70s6k-dependent pathway. Cancer Res 1998;58(22):5239–47.
[62] Arcaro A, Khanzada UK, Vanhaesebroeck B, et al. Two distinct phosphoinositide 3-kinases mediate polypeptide growth factor-stimulated PKB activation. EMBO J 2002;21(19):5097–108.
[63] Blackhall FH, Pintilie M, Michael M, et al. Expression and prognostic significance of kit, protein kinase B, and mitogen-activated protein kinase in patients with small cell lung cancer. Clin Cancer Res 2003;9(6):2241–7.
[64] Tsurutani J, West KA, Sayyah J, et al. Inhibition of the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin pathway but not the MEK/ERK pathway attenuates laminin-mediated small cell lung cancer cellular survival and resistance to imatinib mesylate or chemotherapy. Cancer Res 2005;65(18):8423–32.
[65] Kraus AC, Ferber I, Bachmann SO, et al. In vitro chemo- and radioresistance in small cell lung cancer correlates with cell adhesion and constitutive activation of AKT and MAP kinase pathways. Oncogene 2002;21(57):8683–95.
[66] Belyanskaya LL, Hopkins-Donaldson S, Kurtz S, et al. Cisplatin activates Akt in small cell lung cancer cells and attenuates apoptosis by survivin upregulation. Int J Cancer 2005;117(5):755–63.
[67] Krystal GW, Sulanke G, Litz J. Inhibition of phosphatidylinositol 3-kinase-Akt signaling blocks growth, promotes apoptosis, and enhances sensitivity of small cell lung cancer cells to chemotherapy. Mol Cancer Ther 2002;1(11):913–22.
[68] Warshamana-Greene GS, Litz J, Buchdunger E, et al. The insulin-like growth factor-I receptor kinase inhibitor, NVP-ADW742, sensitizes small cell lung cancer cell lines to the effects of chemotherapy. Clin Cancer Res 2005;11(4):1563–71.
[69] Warshamana-Greene GS, Litz J, Buchdunger E, et al. The insulin-like growth factor-I (IGF-I) receptor kinase inhibitor NVP-ADW742, in combination with STI571, delineates a spectrum of dependence of small cell lung cancer on IGF-I and stem cell factor signaling. Mol Cancer Ther 2004;3(5):527–35.
[70] Ma PC, Tretiakova MS, Nallasura V, et al. Downstream signalling and specific inhibition of c-MET/HGF pathway in small cell lung cancer: implications for tumour invasion. Br J Cancer 2007;97(3):368–77.
[71] Wang WL, Healy ME, Sattler M, et al. Growth inhibition and modulation of kinase pathways of small cell lung cancer cell lines by the novel tyrosine kinase inhibitor STI 571. Oncogene 2000;19(31):3521–8.
[72] Chen YL, Law PY, Loh HH. Inhibition of akt/protein kinase B signaling by naltrindole in small cell lung cancer cells. Cancer Res 2004;64(23):8723–30.
[73] Yeh J, Litz J, Hauck P, et al. Selective inhibition of SCLC growth by the A12 anti-IGF-1R monoclonal antibody correlates with inhibition of Akt. Lung Cancer 2008;60(2):166–74.
[74] Folkes AJ, Ahmadi K, Alderton WK, et al. The identification of 2-(1H-indazol-4-yl)-6-(4-methanesulfonyl-piperazin-1-ylmethyl)-4-morpholin-4-yl-thieno[3,2-d]pyrimidine (GDC-0941) as a potent, selective, orally bioavailable inhibitor of class I PI3 kinase for the treatment of cancer. J Med Chem 2008;51(18):5522–32.
[75] A study of the safety pharmacology of PI3-kinase inhibitor GDC-0941 in combination with paclityxel and carboplatin with or without bevacizumab in patients with advanced non-small cell lung cancer. Study NCT00974584 May 14–June 1, 2010. Available from: www.ClinicalTrials.gov.
[76] A study on the safety and pharmacology of GDC-0941 in combination with erlotinib in patients with advanced solid tumors. Study NCT00975182 May 14–June 1, 2010. Available from: www.ClinicalTrials.gov.
[77] Baird RD, Kristeleit R, Sarker D, Olmos D, Sandhu SK, Yan Y, et al. A phase I study evaluating the pharmacokinetics (PK) and pharmacodynamics (PD) of the oral pan-phosphoinositide-3 kinase (PI3K) inhibitor GDC-0941. J Clin Oncol 2010;28(Suppl.):7s, abstr 2613, Asco Annual Meeting, 2010.
[78] Von Hoff DDLP, Tibes R, Shapiro G, Weiss GJ, Ware JA, et al. A first-in-human phase I study to evaluate the pan-PI3K inhibitors GDC-0941 administered QD or BID in patients with advanced solid tumors. J Clin Oncol 2010;28(Suppl.):7s, abstr 2541, ASCO Annual Meeting, 2010.
[79] Yao E, Zhou W, Lee-Hoeflich ST, et al. Suppression of HER2/HER3-mediated growth of breast cancer cells with combinations of GDC-0941 PI3K inhibitor, trastuzumab, and pertuzumab. Clin Cancer Res 2009;15(12):4147–56.
[80] Junttila TT, Akita RW, Parsons K, et al. Ligand-independent HER2/HER3/PI3K complex is disrupted by trastuzumab and is effectively inhibited by the PI3K inhibitor GDC-0941. Cancer Cell 2009;15(5):429–40.
[81] Salphati L, Wong H, Belvin M, et al. Pharmacokinetic–pharmacodynamic modeling of tumor growth inhibition and biomarker modulation by the novel PI3K inhibitor 2-(1H-Indazol-4-yl)-6-(4-methanesulfonyl-piperazin-1-ylmethyl)-4-morpholin-4-yl-thieno[3,2-d]pyrimidine (GDC-0941). Drug Metab Dispos 2010.
[82] Sutherlin DP, Sampath D, Berry M, et al. Discovery of (thienopyrimidin-2-yl)aminopyrimidines as potent, selective, and orally available pan-PI3-kinase and dual pan-PI3-kinase/mTOR inhibitors for the treatment of cancer. J Med Chem 2010;53(3):1086–97.
[83] Raynaud FI, Eccles SA, Patel S, et al. Biological properties of potent inhibitors of class I phosphatidylinositide 3-kinases: from PI-103 through PI-540, PI-620 to the oral agent GDC-0941. Mol Cancer Ther 2009;8(7):1725–38.
[84] O'Brien C, Wallin JJ, Sampath D, et al. Predictive biomarkers of sensitivity to the phosphatidylinositol 3′ kinase inhibitor GDC-0941 in breast cancer preclinical models. Clin Cancer Res 2010.
[85] Safety study of XL147 in combination with paclitaxel and carboplatin in adults with solid tumors. Study NCT00756847 February 25, 2009–June 1, 2010. Available from: www.ClinicalTrials.gov.
[86] Safety study of XL147 in combination with erlotinib in adults with solid tumors. Study NCT00692640 October 20, 2008–June 1, 2010. Available from: www.ClinicalTrials.gov.
[87] Edelmann G, Bedell C, Shapiro G, Pandya SS, Kwak EL, Scheffold C, et al. A phase I dole-escalation study of XL147 (SAR245408), a PI3K inhibitor administered orally to patients (pts) with advanced malignancies. J Clin Oncol 2010;28(Suppl.):7s, abstr 3004, ASCO Annual Meeting, 2010.
[88] Beuvink I, Boulay A, Fumagalli S, et al. The mTOR inhibitor RAD001 sensitizes tumor cells to DNA-damaged induced apoptosis through inhibition of p21 translation. Cell 2005;120(6):747–59.
[89] Mabuchi S, Altomare DA, Cheung M, et al. RAD001 inhibits human ovarian cancer cell proliferation, enhances cisplatin-induced apoptosis, and prolongs survival in an ovarian cancer model. Clin Cancer Res 2007;13(14):4261–70.
[90] Boulay A, Zumstein-Mecker S, Stephan C, et al. Antitumor efficacy of intermittent treatment schedules with the rapamycin derivative RAD001 correlates with prolonged inactivation of ribosomal protein S6 kinase 1 in peripheral blood mononuclear cells. Cancer Res 2004;64(1):252–61.
[91] Buck E, Eyzaguirre A, Brown E, et al. Rapamycin synergizes with the epidermal growth factor receptor inhibitor erlotinib in non-small-

cell lung, pancreatic, colon, and breast tumors. Mol Cancer Ther 2006;5(11):2676–84.
[92] Majumder PK, Febbo PG, Bikoff R, et al. mTOR inhibition reverses Akt-dependent prostate intraepithelial neoplasia through regulation of apoptotic and HIF-1-dependent pathways. Nat Med 2004;10(6):594–601.
[93] Ramalingam SS, Harvey RD, Saba N, et al. Phase 1 and pharmacokinetic study of everolimus, a mammalian target of rapamycin inhibitor, in combination with docetaxel for recurrent/refractory nonsmall cell lung cancer. Cancer 2010.
[94] Hu L, Hofmann J, Lu Y, et al. Inhibition of phosphatidylinositol 3′-kinase increases efficacy of paclitaxel in in vitro and in vivo ovarian cancer models. Cancer Res 2002;62(4):1087–92.
[95] Soria JC, Shepherd FA, Douillard JY, et al. Efficacy of everolimus (RAD001) in patients with advanced NSCLC previously treated with chemotherapy alone or with chemotherapy and EGFR inhibitors. Ann Oncol 2009;20(10):1674–81.
[96] Milton DT, Riely GJ, Azzoli CG, et al. Phase 1 trial of everolimus and gefitinib in patients with advanced nonsmall-cell lung cancer. Cancer 2007;110(3):599–605.
[97] Price KA, Azzoli CG, Krug LM, et al. Phase II trial of gefitinib and everolimus in advanced non-small cell lung cancer. J Thorac Oncol 2010;5(10):1623–9.
[98] A study of TS-1, cisplatin (CDDP) and RAD001 (Everolimus). Study NCT01096199 March 30–June 1, 2010. Available from: www.ClinicalTrials.gov.
[99] Lee S, Choi EJ, Jin C, et al. Activation of PI3K/Akt pathway by PTEN reduction and PIK3CA mRNA amplification contributes to cisplatin resistance in an ovarian cancer cell line. Gynecol Oncol 2005;97(1):26–34.
[100] Liu LZ, Zhou XD, Qian G, et al. AKT1 amplification regulates cisplatin resistance in human lung cancer cells through the mammalian target of rapamycin/p70S6K1 pathway. Cancer Res 2007;67(13):6325–32.
[101] Wu C, Wangpaichitr M, Feun L, et al. Overcoming cisplatin resistance by mTOR inhibitor in lung cancer. Mol Cancer 2005;4(1):25.
[102] Marinov M, Ziogas A, Pardo OE, et al. AKT/mTOR pathway activation and BCL-2 family proteins modulate the sensitivity of human small cell lung cancer cells to RAD001. Clin Cancer Res 2009;15(4):1277–87.
[103] Stracke S, Ramudo L, Keller F, et al. Antiproliferative and overadditive effects of everolimus and mycophenolate mofetil in pancreas and lung cancer cells in vitro. Transplant Proc 2006;38(3):766–70.
[104] Pandya KJ, Dahlberg S, Hidalgo M, et al. A randomized, phase II trial of two dose levels of temsirolimus (CCI-779) in patients with extensive-stage small-cell lung cancer who have responding or stable disease after induction chemotherapy: a trial of the Eastern Cooperative Oncology Group (E1500). J Thorac Oncol 2007;2(11): 1036–41.
[105] Temsirolimus and vinorelbine ditartrate in treating patients with unresectable or metastatic solid tumors. Study NCT01155258 July 14, 2010. Available from: www.ClinicalTrials.gov.
[106] Metformin and temsirolimus in treating patients with metastatic or unresectable solid tumor or lymphoma. Study NCT00659568 July 14, 2010. Available from: www.ClinicalTrials.gov.
[107] Everolimus, carboplatin, and etoposide in treating patients with small cell lung cancer or other advanced solid tumors. Study NCT00807755 March 25–July 5, 2010. Available from: www.ClinicalTrials.gov.
[108] Combination anticancer therapy of paclitaxel, everolimus for relapsed or refractory small cell lung, cancer. NCT01079481 March 8–July 5, 2010. Available from: www.ClinicalTrials.gov.
[109] Phase II TRial of RAD001 (Everolimus) in previously treated small cell lung cancer. Study NCT00374140 April 3–July 5, 2010. Available from: www.ClinicalTrials.gov.
[110] Tarhini A, Kotsakis A, Gooding W, et al. Phase II study of everolimus (RAD001) in previously treated small cell lung cancer. Clin Cancer Res 2010;16(23):5900–7.
[111] Burris H, Rodon J, Sharma S, Herbst RS, Tabernero J, Infante JR, et al. First-in-human phase I study of the oral PI3K inhibitor BEZ235 in patients (pts) with advanced solid tumors. J Clin Oncol 2010;28(Suppl.):7s, abstr 3005, ASCO Annual Meeting, 2010.
[112] Cho DC, Cohen MB, Panka DJ, et al. The efficacy of the novel dual PI3-kinase/mTOR inhibitor NVP-BEZ235 compared with rapamycin in renal cell carcinoma. Clin Cancer Res 2010.
[113] Maira SM, Stauffer F, Brueggen J, et al. Identification and characterization of NVP-BEZ235, a new orally available dual phosphatidylinositol 3-kinase/mammalian target of rapamycin inhibitor with potent in vivo antitumor activity. Mol Cancer Ther 2008;7(7):1851–63.
[114] Serra V, Markman B, Scaltriti M, et al. NVP-BEZ235, a dual PI3K/mTOR inhibitor, prevents PI3K signaling and inhibits the growth of cancer cells with activating PI3K mutations. Cancer Res 2008;68(19):8022–30.
[115] Bhatt AP, Bhende PM, Sin SH, et al. Dual inhibition of PI3K and mTOR inhibits autocrine and paracrine proliferative loops in PI3K/Akt/mTOR-addicted lymphomas. Blood 2010;115(22):4455–63.
[116] Zitzmann K, Rüden J, Brand S, et al. Compensatory activation of Akt in response to mTOR and Raf inhibitors – a rationale for dual-targeted therapy approaches in neuroendocrine tumor disease. Cancer Lett 2010;295(1):100–9.
[117] Dose defining study for MK-2206 combined with gefitinib in non-small cell lung cancer (NSCLC). Study NCT01147211 June 17–July 5, 2010. Available from: www.ClinicalTrials.gov.
[118] Hirai H, Sootome H, Nakatsuru Y, et al. MK-2206, an allosteric Akt inhibitor, enhances antitumor efficacy by standard chemotherapeutic agents or molecular targeted drugs in vitro and in vivo. Mol Cancer Ther 2010;9(7):1956–67.
[119] White DA, Schwartz LH, Dimitrijevic S, et al. Characterization of pneumonitis in patients with advanced non-small cell lung cancer treated with everolimus (RAD001). J Thorac Oncol 2009;4(11):1357–63.
[120] Duran I, Siu LL, Oza AM, et al. Characterisation of the lung toxicity of the cell cycle inhibitor temsirolimus. Eur J Cancer 2006;42(12):1875–80.
[121] Cherrin C, Haskell K, Howell B, et al. An allosteric Akt inhibitor effectively blocks Akt signaling and tumor growth with only transient effects on glucose and insulin levels in vivo. Cancer Biol Ther 2010;9(7):493–503.
[122] Luo Y, Shoemaker AR, Liu X, et al. Potent and selective inhibitors of Akt kinases slow the progress of tumors in vivo. Mol Cancer Ther 2005;4(6):977–86.
[123] Crouthamel MC, Kahana JA, Korenchuk S, et al. Mechanism and management of AKT inhibitor-induced hyperglycemia. Clin Cancer Res 2009;15(1):217–25.
[124] Meuillet EJ, Ihle N, Baker AF, et al. In vivo molecular pharmacology and antitumor activity of the targeted Akt inhibitor PX-316. Oncol Res 2004;14(10):513–27.
[125] Garofalo RS, Orena SJ, Rafidi K, et al. Severe diabetes, age-dependent loss of adipose tissue, and mild growth deficiency in mice lacking Akt2/PKB beta. J Clin Invest 2003;112(2):197–208.
[126] Brachmann SM, Ueki K, Engelman JA, et al. Phosphoinositide 3-kinase catalytic subunit deletion and regulatory subunit deletion have opposite effects on insulin sensitivity in mice. Mol Cell Biol 2005;25(5):1596–607.
[127] Foukas LC, Claret M, Pearce W, et al. Critical role for the p110alpha phosphoinositide-3-OH kinase in growth and metabolic regulation. Nature 2006;441(7091):366–70.
[128] Jou ST, Carpino N, Takahashi Y, et al. Essential, nonredundant role for the phosphoinositide 3-kinase p110delta in signaling by the B-cell receptor complex. Mol Cell Biol 2002;22(24):8580–91.
[129] Okkenhaug K, Bilancio A, Farjot G, et al. Impaired B and T cell antigen receptor signaling in p110delta PI 3-kinase mutant mice. Science 2002;297(5583):1031–4.

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